



# 1

## Introduction

The *Cry* and *Per* mutant studies focussed on the full phase response curves for brief light pulses (chapter 5), the behavioural adjustment to changing photoperiods (chapter 7) and the rhythmicity in constant illumination (chapter 8). For all these conditions the molecular E-M model yielded robust predictions which we could test. In addition, the collaboration with the group of Howard Cooper in Lyon (R.A.Hut, M.M.Okolewicz) made it possible to study a potential corollary of the model. It is known that the induction of the immediate early gene *cFos* in the suprachiasmatic nucleus by light is associated with behavioural phase resetting (e.g., Wollnik et al. 1995). One might expect that component oscillators control their own light input and thereby affect whether *cFos* is induced by light depending on time of day. Although this is not necessarily a test of the E/M model we carried out this study in the mutant and wildtype strains. This is extensively described in chapter 6.

The final two chapters provide an overview of where we now stand on the issue of the E/M system. Chapter 9 provides a summary of most of the experiments done, while chapter 10 gives primarily a perspective on further ideas and future work. While our experiments have provided some limited support for the opposite effects of *Per1* and *Per2* in the system which snugly fit into the E/M hypothesis, much of the *Cry* knockout studies failed to confirm the predictions. Yet we retain some faith in the concept, and that faith has recently been reinforced by the highly elegant unraveling of the pacemaker in *Drosophila melanogaster* which turns out to consist of two groups of neurons, one responsible for the behavioural anticipation of dawn, one for the behavioural anticipation of dusk (Stoleru et al. 2004; Grima et al. 2004; Schwartz 2004). One biological problem, one solution ?

## Entrainment to the natural light/dark cycle

The initial inspiration for the project that forms the basis of this dissertation was the ‘case of the European ground squirrel’ the problem that the classic PRC model for entrainment could not explain the entrainment of the European ground squirrel (*Spermophilus citellus*) to the natural light/dark cycle (Hut et al. 1999). The enigma of an accurately entrained rhythm in animals that virtually never in their life observe dawn or dusk led to the concept that light does much more than generate discrete phase shifts to correct for aberrations in the period, and rather causes biological clocks to tick at exactly 24-h periods. Careful measurements of both a phase response curve (PRC) and a period response curve ( $\tau$ RC), and model simulations (Beersma et al. 1999) suggested an important role for  $\tau$  responses in the entrainment of the species to natural light.

Human circadian behaviour is comparable to the circadian behaviour of the ground squirrels. We too determine our light exposure by behaviour. In daytime we lock ourselves up in offices that are illuminated with just a fraction of outside sunlight, and in the evening we switch on artificial light until we decide it is time to sleep. This behaviour results in a very irregular light exposure pattern with a ‘shoulder’ at the end of the day by artificial illumination. Yet human circadian clocks remain perfectly entrained to the external time, with rather small variance in the phase of different chronotypes for instance (Roenneberg et al. 2003b). A logical step was to see whether circadian entrainment of humans to these erratic light patterns would benefit from period responses as well. In **chapter 2** we present the results from a theoretical simulation study for human entrainment, comparable to the simulation for entrainment of European ground squirrels (Beersma et al. 1999), on basis of actual human illumination patterns collected around the equinox.

Even if changes in circadian period enable the species to entrain more precisely to the natural light/dark cycle by only subtle adjustments both in phase and pacemaker velocity, these adjustments still have to be made on the basis of frequent and recurring Zeitgeber time cues. Since the ground squirrels emerge above ground long after twilight at dawn, and retreats underground hours before dusk, rapid changes in light intensity cannot be perceived (Hut et al. 1999). The only change in light intensity they can observe is a 1 log unit decrease in light intensity by the end of the afternoon. Are such weak and unreliable signals really used for entrainment, or does reduced light chase the animals into their burrows, such that they themselves provide a secondary timing cue by their own behaviour. We needed a field experiment to resolve this question: darken the meadow at an other time of day, and see whether the animals respond. On August 11, 1999, nature itself provided the experiment: test whether a light intensity drop caused the animals to retreat. We used the solar eclipse that darkened a natural population of “soslirks” near Vienna in the middle of the day for a few minutes. The results of this natural experiment are described in **chapter 3**.

### The dual oscillator model

Optimizing the behaviour of different species, such as the European ground squirrel, demands a continuous adjustment of the endogenous circadian program to changing day length. Many physiological processes recur on an annual basis and depend on accurate measurement of the time of year. In mammals, the duration of the subjective day and night is stored by the SCN (Sumová et al. 1995; Sumová and Ilnerová 1998; Jagota et al. 2000; Mrugala et al. 2000). The mechanism responsible for day length coding is not clear. The theory of Pittendrigh provides a theoretical explanation that enables a master circadian oscillator to encode day length. It proposes that the master pacemaker consists of two internally coupled oscillators with differential properties. One oscillator (E for evening) is supposedly decelerated by light, and the other oscillator (M for morning) is accelerated by light, thereby tracking dusk and dawn, respectively. This model yields specific predictions towards a pacemaker that has a defect in either the E or the M oscillator.

### The molecular clock and the EM model

The recent unraveling of the molecular system behind the circadian pacemaker makes it possible to study specific properties and function of molecular components within the core of the circadian clock. With the availability of mice with mutations in core clock genes, mutant circadian phenotypes can be assessed which in turn elucidate properties of the circadian system.

The phenotype of *mPer1<sup>Brdm1</sup>*, *mPer2<sup>Brdm1</sup>*, *mCry1<sup>-/-</sup>* and *mCry2<sup>-/-</sup>* mutant mice did show signs of a pacemaker that has either a defective E or a defective M oscillator (van der Horst et al. 1999; Albrecht et al. 2001) or of a pacemaker that has both a defective E and M oscillator by double mutations (Zheng et al. 1999; van der Horst et al. 1999). This led to the proposition of a molecular basis for the original EM hypothesis (Daan et al. 2001). This provided testable predictions with respect to the circadian behaviour of mice with these mutations regarding phase resetting, rhythmicity, circadian phenotype in constant light and the response to changing photoperiod. These predictions and their experimental tests are the focus of most of the rest of the thesis. Most of the work was done with *mPer1<sup>Brdm1</sup>* and *mPer2<sup>Brdm1</sup>* mice, available through the help of Dr. U. Albrecht and the *mCry1<sup>-/-</sup>* and *mCry2<sup>-/-</sup>* knockouts mice, given to us by Dr.G.T.J. van der Horst. It is these genes in particular to which a specific role was assigned in the E-M model, viz. that *per1* and *cry1* would be part of the M component, and *Per2* and *Cry2* part of the E component.

I made one interesting digression using mice with a mutation of the *clock* gene (Vitaterna et al. 1994), another gene belonging to the central circadian clock machinery. In anticipation of the studies with constant light in the four *Per* and *Cry* mutant lines, we exposed mice with mutant clock alleles to LL and observed that most animals become rhythmic under conditions where wildtypes have lost their self-sustained rhythmicity (chapter 4). This turned out later to be true also of *per2*-mutants.

## INTRODUCTION

Endogenous self sustained circadian organization is one of the fundamental properties of life on earth. The circadian system is a well preserved function that subserves the optimal timing of physiological processes and organization of behaviour. It enables organisms to anticipate periodically recurring daily events. It is present in nearly all phyla, from unicellular organisms to vertebrates. They display the capacity for self-sustained rhythmicity when deprived of any external clue denoting the time of day. Yet this self-sustained capacity is a property deriving its function for the fitness of the organism from its behaviour under entrainment. Entrainment provides the true key to both function and mechanism of circadian organisation. In complex organisms the function of entrainment is often condensed in central nervous pacemakers that both channel the information on the day outside via often specialized photoreceptors to the body and coordinate and synchronize the multitude of oscillations in cells and tissues. In mammals, the master circadian pacemaker is located in the suprachiasmatic nuclei (SCN) of the hypothalamus (Ralph et al. 1990). The SCN both regulates the distribution of the activity throughout the day or night and drives peripheral oscillators in the rest of the body.

The principal theory on entrainment of pacemakers by light is due to Pittendrigh (1981 and before), and states that the endogenous rhythm has a cycle slightly deviant from 24 h, a deviation that is corrected for by instantaneous resets or phase shifts in response to light every single day. The sign and magnitude of the phase shifts are phase dependent and thereby yield predictable stable phases of the entrained system with the outside zeitgeber, or the perfect match between Internal Time and External Time. This view of phase dependent resets fully suffices to understand global patterns of entrainment in simple rectangular laboratory LD 12:12 conditions. It failed, however, to explain the dynamics of behaviour e.g., of nocturnal mammals under changing photoperiods (Pittendrigh and Daan 1976b), or that of diurnal mammals out in nature that remain entrained without ever observing dawn or dusk (Hut et al. 1999). These behaviours call for greater complexity of the system than assumed in the simple phase-only model of instantaneous discrete shifts. They led Pittendrigh himself to the formulation of a more complex theory in which the pacemaker is composed of two different elements that together adjust flexibly to the varying daylength outside (Pittendrigh and Daan 1976c). The new potential offered by the genomics developments now calls for renewed interest in the problem of entrainment by light. Many of the old behavioural protocols applied to intact wild animals can now be applied to animals with genetically engineered clock systems to further probe into the their functional organization. This thesis aims to contribute to the understanding of the complexity of circadian entrainment.