Network properties of the mammalian circadian clock

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Chapter 2

Mechanisms of the mammalian clock

The master circadian clock in mammals is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN consist of two bilaterally paired nuclei situated on opposite sides of the third ventricle, just above the optic chiasm (figure 2.1) (Klein et al., 1991).

The SCN were initially identified as the mammalian circadian clock in lesion studies. When the SCN was lesioned from the brain, a loss of rhythmicity in behaviour was observed (Moore and Eichler, 1972;Stephan and Zucker, 1972). Transplantation studies strengthened this hypothesis. When SCN tissue was transplanted in animals without an SCN circadian rhythms returned, also when the transplanted tissue was from a completely different animal strain (Ralph et al., 1990). In addition, electrical activity studies showed that the SCN has circadian rhythms, also when kept in constant darkness (Groos and Hendriks, 1982). When techniques became more refined, circadian rhythmicity profiles in electrical activity of single SCN neurons could also be obtained (Welsh et al., 1995;Liu et al., 1997;Herzog et al., 1998;Honma et al., 1998). This indicated that SCN neurons have an endogenous circadian rhythm.

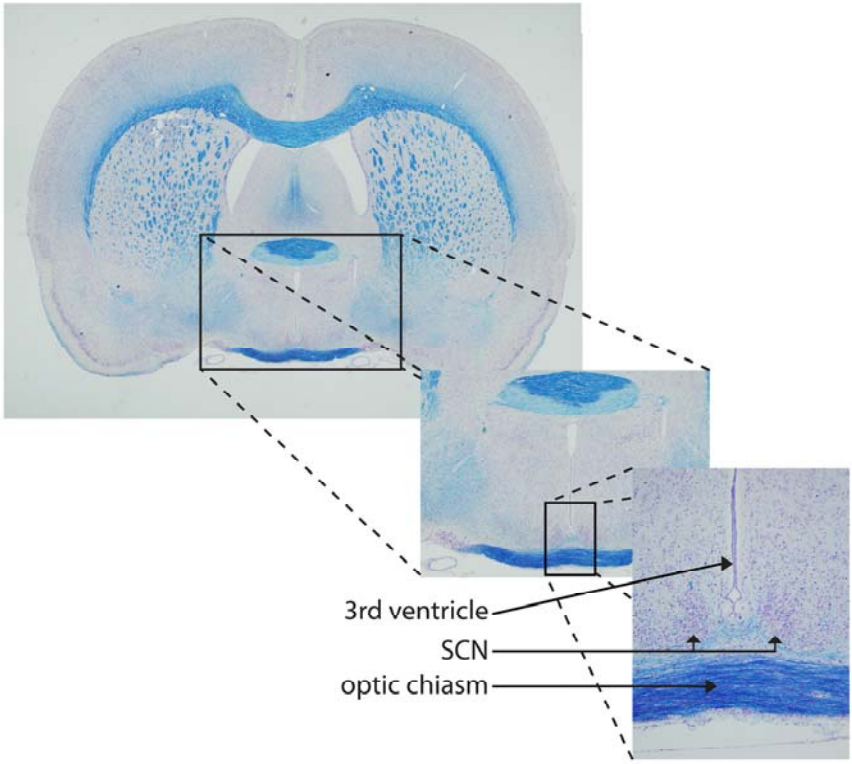

Figure 2.1 Brain of a rat containing both suprachiasmatic nuclei on opposite sides of the third ventricle, just above the optic chiasm.

2.1 Intracellular feedback loops

Underlying the endogenous rhythms of the SCN neurons are transcriptional translational feedback loops. The main genes that are involved in these regulatory loops are Clock, Bmal1, the three period genes (Per1, Per2, and Per3) and the two cryptochrome genes (Cry1 and Cry2) (see figure 2.2).

A rhythmic expression of Bmal1 enables the formation of CLOCK and BMAL1 protein complex. This complex, while in the cell nucleus, activates the transcription of the period and cryptochrome genes into mRNA. Liposomes then translate the mRNA into the PER and CRY proteins. These proteins form heterodimers (complexes with each other), enabling localization into the cell nucleus, where complexes containing CRY1 and CRY2 protein then inhibit the activity of the CLOCK- BMAL1 complex,

and with it their own expression. This is a negative feedback loop (Reppert and Weaver, 2002;Lowrey and Takahashi, 2004).

The CLOCK-BMAL1 complex also activates transcription of Rev-Erbα. The resulting REV-ERBα protein then represses the transcription of Bmal1. When the complexes containing PER2 have entered the nucleus, PER2 may be involved in the activation of Bmal1 expression. This is a positive feedback loop. Note that both loops are interlocked, because of the CLOCK-BMAL1 protein complex (Reppert and Weaver, 2002;Lowrey and Takahashi, 2004).

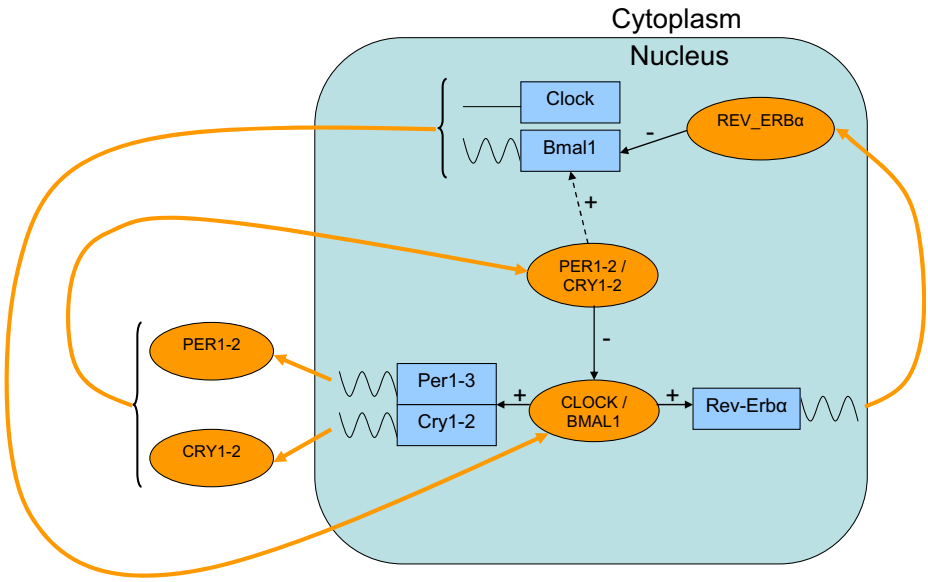

Figure 2.2 Simplified model for the molecular transcriptional / translational feedback loop underlying endogenous rhythms in SCN clock cells. Important clock genes are Bmal1, Clock, Per1-3, Cry1-2 and Rev-Erbα, where Clock is the only gene that is not rhythmically expressed. The genes are depicted in the figure as blue squares. These clock genes are expressed in the nucleus and transformed to proteins in the cytoplasm (BMAL1, CLOCK, PER1-3, CRY1-2 and REV-ERBα). There they form complexes that can re-enter the nucleus to perform its excitatory or inhibitory task (BMAL1/CLOCK, PER/CRY, the complex for REV_ERBα is unknown at this time). The protein and protein complexes are orange circles. BMAL1/CLOCK stimulates expression of Per, Cry and Rev-Erbα, complexes containing CRY inhibit the activity of the BMAL1/CLOCK complex, and the complex containing REV-ERBα represses the expression of Bmal1 (all denoted by black arrows where a + sign means stimulating and a – sign inhibitory influence). Complexes containing PER2 may be involved in activation of Bmal1 expression (black dashed arrow).

While the Clock transcription remains constant, the Bmal1 expression is rhythmic. The transcription of Bmal1 peaks in the middle of the circadian night. Per1 expression is at its peak at the beginning of the subjective day, while Per2 expression peaks at the end of the subjective day, just like Cry1 and Cry2. Per1 and Per2 are believed to be the most important genes involved in phase adjustment to entrain to the light dark cycle (Lowrey and Takahashi, 2004).

2.2 How to measure the rhythm of the clock

The rhythms of the SCN can be measured in behaviour, in multiunit output, in single unit output or in gene expression profiles, using a wide range of approaches. Each method has its own advantages and disadvantages. For example, some methods are better suited for long-term measurements, some methods are especially suited for measurements at a very small timescale, and other methods are suited to do very precise measurements (Aton and Herzog, 2005).

Behavioural rhythms can be measured using running wheels or by measuring drinking activity. The rhythm of the clock can be determined by measuring clock controlled hormone levels in blood samples. Technological advances have allowed also to measure directly from the SCN. This last method is a powerful method because of the direct way of measuring the SCN activity.

Electrical activity in the SCN can be measured, both *in vivo*, where an electrode is implanted in the central nervous system of an animal, as well as *in vitro*, in brain slices, where the SCN is recorded in relative isolation. Electrodes are used to record the spikes. A computer program counts the number of action potentials that exceed a noise-threshold, either for one neuron using patch clamp techniques, or for neuronal populations using extracellular recordings which do not damage the neurons that are measured.

Numerous bioluminescence and fluorescence markers are nowadays available to measure in one neuron the expression of genes, protein products, or intracellular messengers, such as calcium concentrations. Animal models have been created that have a mutation to react to a specific marker, and when concentrations of a particular gene or substance is high, the marker is

also abundantly present in the SCN and this concentration can be visualized with the aid of a camera. Sometimes, the mRNA levels are measured using these methods and in other occasions protein levels are used.

One can also measure the rhythms of cultured SCN neurons. In this case, neuronal populations of SCN cells are transferred to dishes. In these cell cultures it is easier to measure electrical activity and gene expression in the single cells as the individual cells can be better visualized. Cultures are also the preparation of choice when electrophysiological recordings are performed with microelectrode arrays. Note that these cells are not in a 'physiological' environment, which means that the natural network of cells has been disturbed.

2.3 Networks of oscillating neurons

The electrical activity patterns and gene expression profiles of single SCN neurons that are connected in a network have been compared to those measured in isolated or dispersed SCN neurons. The average period length was similar between the neurons with and without a network. However, the variance of the periods was much wider in the isolated neurons, compared to the connected neurons in a network (Herzog et al., 2004). It has become apparent that the interaction between SCN neurons improves the precision of the circadian rhythm. In order for the complete SCN to produce a consistent rhythmic output, the rhythms of the individual neurons must be synchronized, and some communication between the neurons is necessary to realize synchronization (Herzog et al., 1998;Honma et al., 1998;Herzog et al., 2004;Aton and Herzog, 2005).

To examine the synchronization between neurons it is important to realize that the SCN is not one homogeneous population of neurons, and that not all neurons are identical (figure 2.3). The SCN consist of two nuclei, one to the left of the third ventricle and the other to the right of the third ventricle. Each nucleus contains about 8,000 – 10,000 neurons. The neurons in both nuclei are organized in different functional subregions and serve different functions in the regulation of the circadian clock (Antle and Silver, 2005;Aton and Herzog, 2005). This means that there is a heterogeneous population of neurons present in the SCN.

A common distinction that is made for functional subregions of the SCN is between the dorsal SCN (also called *shell*) and the ventral SCN (or historically named *core*). In the rat, a clear distinction between these regions exists anatomically (van den Pol, 1991), whereas in the mouse SCN this anatomical distinction is less clear. However, in the mouse, the functionality of ventral and dorsal neurons still exists (Vansteensel et al., 2008).

The ventral SCN receives most of the light input fibers and mainly contains neurons that produce vasoactive intestinal polypeptide (VIP) or gastrin-releasing peptide (GRP). The dorsal SCN receives input from non-visual cortical and subcortical regions and light information via the ventral SCN. The dorsal SCN mainly consists of neurons producing arginine vasopressin (AVP) (Moore et al., 2002). Other studies also show that there must be a connection from dorsal to the ventral SCN (Albus et al, 2005). Figure 2.4 shows the location of the AVP, VIP and GRP in the dorsal and ventral SCN.

2.4 Properties of the clock: seasonality

A number of attributes of the circadian clock are thought to be produced at the network level, and do not originate at the molecular level. Seasonality is one example of a network driven property of the clock. Seasonal changes have a considerable influence in the lives of many organisms. Reproduction in different organisms is driven by seasonality (plants: Carre, 2001; birds: Dawson et al., 2001; fungi: Roenneberg and Merrow, 2001; mollusks: Wayne, 2001; mammals: Messager et al., 2000). Other mechanisms that are also under influence of the seasons are stem and leaf elongation in plants (Carre, 2001), molt and song behaviour in birds (Dawson et al., 2001), and pelage, appetite and body weight in mammals (Messager et al., 2000).

The most predictable indicator for the different seasons is the change in day length. In summer, the days are longer and the nights shorter, while in winter, vice versa, the days are shorter and the nights longer. In mammals, an impressive amount of research has been carried out on photoperiodism. Changes in photoperiod are observed in locomotor behaviour, melatonin levels in the pineal gland, gene expression profiles and electrical activity rhythms in the SCN (Goldman, 2001;Johnston, 2005;Meijer et al., 2007).

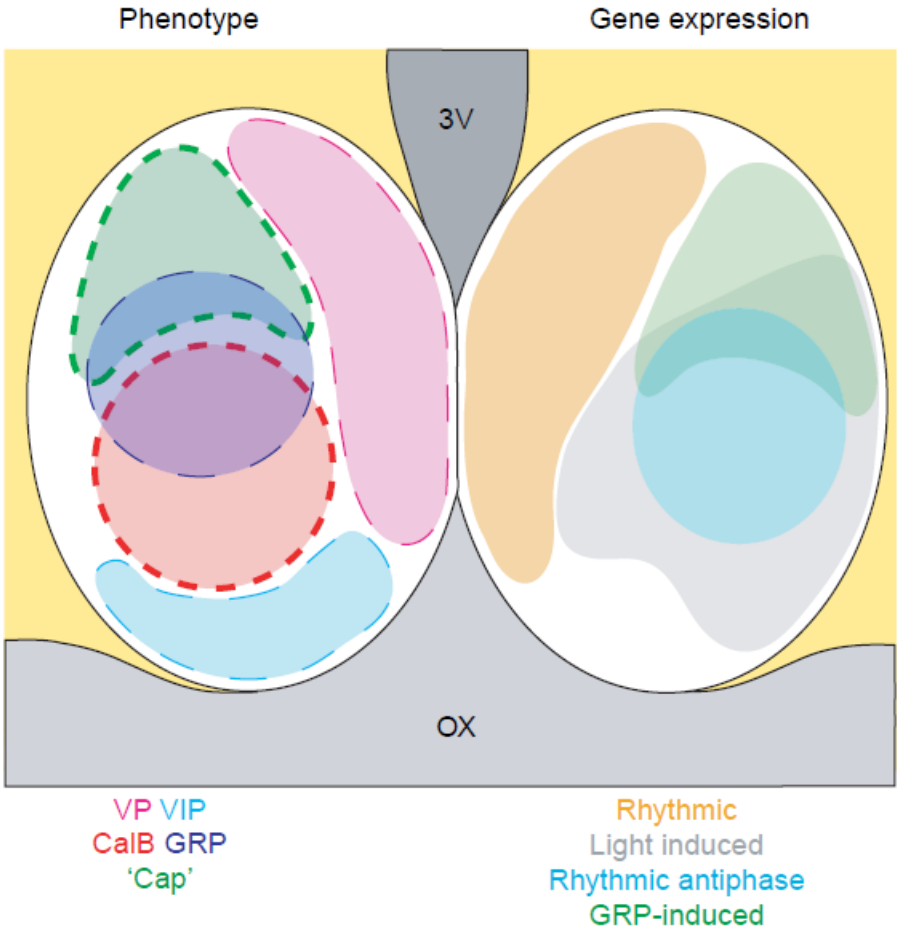

Figure 2.3 Heterogeneous SCN in hamster. The left depicts the different phenotypic subregions in the hamster SCN. In the dorsomedial part of the SCN, vasopressin (VP)-expressing cells (pink) can be found. In the ventral part of the SCN, vasoactive intestinal polypeptide (VIP)-containing cells (light blue) are present. Immediately dorsal to the VIP cells lie calbindin (CalB)-expressing cells (red). The phenotype of the 'cap' cells (green) has not yet been identified, but lie dorsal to the CalB cells, while the gastrin-releasing peptide (GRP)-expressing cells (dark blue) overlap with the CalB and the 'cap' regions. In the right SCN regions are shown that depend on the expression of the Period genes. Per gene expression can either be rhythmic (pale orange region), light-induced (gray region) or follow GRP administration (green region). The blue region contains cells expressing Per in antiphase to the rhythmic Per gene expression. However, these cells are only found in mice and rats, not in hamsters. (Reprinted from TRENDS in Neuroscience, Vol. 28 No. 3, Antle and Silver, Orchestrating time: arrangements of the brain clock, 145-151, Copyright 2005, with permisson from Elsevier.)

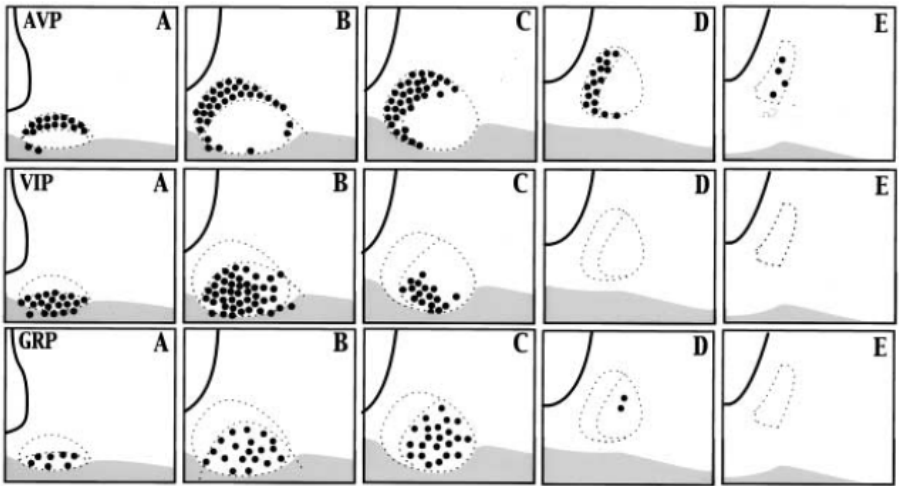

Figure 2.4 Drawings of successive rostral to caudal levels (A-E) depicting the distribution of peptide phenotype of SCN neurons. Arganine vasopressin (AVP) is mainly produced in the dorsal part of the SCN, while ventral neurons mainly produce vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) (With kind permission from Springer Science+Business Media: Cell and Tissue Research, Suprachiasmatic nucleus organization, Vol. 309, 2002, 89-98, Moore, R.Y., Speh, J.C., Leak, R.K., part of figure 2).

The behaviour of rats and mice and hamsters can be observed by recording running wheel activity. Rats, mice and hamsters are active during the night, and their behavioural periods are in the night. It has been shown that short photoperiods leads to longer activity profiles while long day lengths lead to compressed periods of activity (Refinetti, 2002;Weinert et al., 2005). Also in Syrian and Siberian hamsters, the wheel running activity period increased in short day lengths (Elliott and Tamarkin, 1994;Nuesslein-Hildesheim et al., 2000). The total amount of behavioural activity does not increase in short photoperiods but the activity is spread out over a longer time interval (Refinetti, 2002).

The rhythms of pineal *N*-acetyltransferase activity, which is responsible for the nighttime synthesis of melatonin in the pineal gland, are also affected by day length. The melatonin level is high during the night en low during the day. Therefore, in mice (Weinert et al., 2005) and in Syrian hamsters (Elliott and Tamarkin, 1994) the phase and duration of the pineal melatonin peak is strongly correlated to the phase and duration of locomotor activity. Locomotor activity as well as the rhythms of pineal *N*-acetyltransferase

activity are both controlled by the SCN (Klein and Moore, 1979). This indicates that the SCN is directly under the influence of photoperiod (Sumova et al., 2000).

The output of the SCN, measured as locomotor activity or melatonin levels, thus shows a clear distinction between long and short day lengths. The genes that are thought to be involved in the transcriptional-translational feedback loops that compose the molecular clockwork are also affected by photoperiod. Per1 mRNA level rise occurs in the morning. In long photoperiods, the duration of the high level of Per1 mRNA is extended, while the amplitude is lower than under short photoperiods (Messager et al., 1999;Messager et al., 2000;Steinlechner et al., 2002;Sumova et al., 2003;Tournier et al., 2003). The amplitude of the expression of Per2 is higher on short days than on long days, similar to Per1 expression, but the duration of the peaks under both photoperiods does not substantially differ (Steinlechner et al., 2002;Tournier et al., 2003). Similar results were found for the level of PER1 and PER2 protein (Nuesslein-Hildesheim et al., 2000). Per3 mRNA levels do not differ in amplitude but in duration between short and long photoperiods. In short photoperiods the peak duration is not as long as in long photoperiods (Tournier et al., 2003). Cry1 mRNA rises at dawn. In a long photoperiod its phase was advanced compared to a short photoperiod. However, the duration of the Cry1 mRNA level did not change. Thus, the phase of the Cry1 mRNA rhythm only advanced in a long photoperiod without influencing the duration of the waveform. The amplitude in a short photoperiod did appear to be larger, similar to Per1 and Per2 mRNA (Sumova et al., 2003;Tournier et al., 2003). The duration and amplitude of the nightly peak of Cry2 expression decreases during short photoperiods (Tournier et al., 2003). The Bmal1 mRNA level is high during the dark period. In short photoperiods, the decrease in the morning shifts phase, the duration expands, and the amplitude decreases. This is opposite to what is observed in the daytime-active mPer1 rhythm (Sumova et al., 2003;Tournier et al., 2003). The expression of Clock is constantly high in long photoperiods, while in short photoperiods, a rhythmic pattern emerges (Sumova et al., 2003;Tournier et al., 2003). It is apparent that the clock genes all respond differently to changes in day length.

At the network level photoperiodic differences can be observed in the electrical activity pattern. The electrical activity level is high during the day and low during the night. In vitro and in vivo electrophysiological recordings show long activity during long photoperiods and short activity peaks in short photoperiods. In Syrian hamsters, the electrical activity peak, measured in vitro, is twice as broad as in short photoperiods. However, the duration of the electrical activity in 'normal' photoperiod (12:12) is not longer than in short days. It appears that the critical photoperiod of hamsters (which is 12.5 h) brings about a sudden transition towards longer electrical activity peaks (Mrugala et al., 2000). In vivo and in vitro recordings in mice show short electrical activity patterns in short photoperiod and long patterns in long photoperiod (VanderLeest et al., 2007). In rats the electrical activity pattern measured in vitro increases in width in long photoperiods. Furthermore, the amplitude of the peak decreases and is phase advanced. In short photoperiods the electrical activity pattern was narrower, with increased amplitude and delayed phase with regard to a 12 h photoperiod (Schaap et al., 2003). In rats and mice, as opposed to hamsters, no sign of a sudden transition towards a longer electrical activity peak could be identified.

In subpopulation and single cell analysis of electrical recordings it was shown that the distribution of small subpopulations of neurons in long photoperiods were more dispersed over the 24 h cycle than in short photoperiods (VanderLeest et al., 2007). This serves as an indication for a tighter coupling in the network in short day lengths and a looser coupling between the neurons of the SCN in long photoperiods.

In summary, photoperiod has a profound effect on the duration, the amplitude and the phase of many parts of the circannual and circadian system. To account for photoperiod, different models have been proposed. In the model proposed by Aschoff (1960) the parametric effects of light were emphasized. This means that the duration and intensity of light was taken to be important and resulted in a phase response curve (Aschoff, 1960;Wever, 1972). Nowadays this model is often referred to as the external coincidence model (Tauber and Kyriacou, 2001;Dawson et al., 2001). An alternative model was suggested by Pittendrigh and Daan (Pittendrigh and Daan, 1976b). In this model non-parametric effects of light were assumed to

determine photoperiodic encoding. The transitions from dark to light during dawn and from light to dark during dusk were considered to be the most important clues. This model is also referred to as the E-M model (evening-morning model) or the internal coincidence model (Pittendrigh and Daan, 1976b;Daan and Berde, 1978;Tauber and Kyriacou, 2001;Dawson et al., 2001;Elliott and Tamarkin, 1994;Sumova et al., 1995;Vuillez et al., 1996;Schwartz et al., 2001;Steinlechner et al., 2002;Weinert et al., 2005). Daan et al. (2001) tried to relate this model to available and new molecular findings. It was proposed that the Per1 mRNA levels reflected the timing of the M oscillator, while the Per2 mRNA levels determined the E oscillator (Daan et al., 2001). However, with the findings of photoperiodic effects on different clock genes, this model is no longer accepted (Sumova et al., 2003;Tournier et al., 2003).

2.5 Properties of the clock: jet lag

Another example of an alleged network driven property of the SCN is the phenomenon of jet lag, which is associated with sudden shifts in the phase of the light period. The circadian clock in mammals has an endogenous rhythm of approximately 24 hours. For humans this is somewhat longer, while for rats and mice this rhythm is a bit shorter. In normal circumstances, the daily light-dark cycle adjusts the clock every day to its 24 hour cycle by the induction of small phase shifts. Mammals experience no problems when such small corrections happen at a daily basis. However, when sudden larger shifts in phase take place, for instance as a consequence of a transatlantic flight, jet lag problems like fragmented sleep, premature awakening, excessive sleepiness and a decrement in performance can occur (Waterhouse et al., 2007;Reddy et al., 2002). Jet lag phenomena take place because the different circadian rhythms in the body are not (yet) synchronized to the new time zone (Waterhouse et al., 2007;Takahashi et al., 2002). The same phenomena can also occur with rotational shift work or sleep disturbance (Reddy et al., 2002).

The severity of jet lag increases with the number of time zones crossed and flights to the east cause more problems than westward flights (Waterhouse et al., 2007). Eastward travelers experience a shorter total sleep

time, are more active in their sleep and this sleeping phase is shifted towards earlier hours. Westward travelers experience a sleep phase shift towards later hours, but experience less sleeping problems (Takahashi et al., 2002).

The SCN is directly influenced by the daily light dark cycle, and is therefore directly affected by a sudden change in this regime. The SCN is supposed to re-entrain all peripheral oscillators to the new time regime (Yamazaki et al., 2000). Peripheral oscillators are for example rhythms in body temperature, pineal melatonin levels, plasma hormone concentrations, and organs, like skeletal muscle, liver and lung (Waterhouse et al., 2007;Yamazaki et al., 2000). These rhythms should not immediately be perturbed by external factors so that the system is able to retain a stable phase in a noisy environment. However, this protection against unrequired phase shifts also causes the problems associated with jet lag (Waterhouse et al., 2007).

In a laboratory, jet lag situations can be simulated by advancing the light phase (mimicking eastward flights) or delaying the light phase (simulating westward flights). Using these schemes, effects of phase delays and advances on different mechanisms, such as gene expression and electrical activity, in the SCN have been assessed.

After a phase delay of 6 hours, which is comparable to a flight from Amsterdam to New York, behavioral rhythms entrain very rapidly to the new regime. The transition takes less than two days. After a phase advance however, comparable to the return flight mentioned, the behavioral rhythm takes at least six days before it is completely shifted to the new phase, which emphasizes the difference between westward and eastward flight (Yamazaki et al., 2000;Reddy et al., 2002). When using a different protocol, similar differences were found between a delay and an advance of the light-dark cycle (Albus et al., 2005;Vansteensel et al., 2003;van Oosterhout et al., 2008). What becomes clear is that a behavioral phase shift due to an advance of the light-dark cycle is more difficult than due to a delay of the light-dark cycle.

Different genes have been assessed after a phase advance or delay of the light cycle. The expression of Per1 showed a rapid phase shift immediately after a delay or an advance (Reddy et al., 2002;Nagano et al.,

2003;Yamazaki et al., 2000;Vansteensel et al., 2003). In different regions of the SCN, the response appeared to be different. In the ventral part of the SCN the shifts were rapid, while in the dorsal part the shift took much longer, and an advance was more difficult than a delay (Nagano et al., 2003). Per2 expression showed the same characteristics as Per1 (Reddy et al., 2002;Nagano et al., 2003). For delays, Cry1 gene expression also showed the same characteristics, but for phase advances it took longer before Cry1 was fully re-entrained (Reddy et al., 2002).

At the network level, in vitro electrical activity measurements showed two concurrent peaks following a delay of the light-dark cycle of 6 hours. The electrical activity in the ventral SCN appeared to be shifted immediately to the new phase, while in the dorsal SCN, the shift was completed only after 6 days (Albus et al., 2005). In vitro electrical activity measurements after 6 hour advances of the light-dark cycle showed an immediate shift of about 3 hours. When the slice was prepared 6 days after the shift, the phase of the SCN was back at the old light-dark regime (so no phase shift did take place in the end). In vivo electrical activity showed no phase shift at all, indicating that the dorsal SCN does not shift and prevents the ventral SCN from shifting (Vansteensel et al., 2003). For mice, similar results are observed after phase advances of the light-dark cycle. The in vitro recordings show immediate phase shifts on the first day, while the shift obtained in in vivo recordings is only very small (van Oosterhout et al., 2008).

In conclusion, it is clear that regional differences in functionality of the SCN lead to a desynchronization of (groups of) neurons after a sudden large shift of the light-dark cycle, leading to jet lag.

2.6 Properties of the clock: arrhythmicity

Jet lag phenomena are caused by different oscillatory mechanisms of the body that run out of phase with each other. We have seen which profound problems this can cause and that only after the SCN and the peripheral oscillators are resynchronized with each other, these jet lag problems disappear.

Another well known example that disrupts behavioral and physiological rhythmicity is the exposure to constant light (LL). Hamsters show peculiar

behavior when put into a regime where the light is constantly on and no dark period is given to these night-active animals. A number of animals in such a constant light (LL) regime begin to show a so-called 'split' rhythm, which is a rhythm of 6 hours of activity and 6 hours of inactivity. So two 12-hour rhythms in one circadian day (Pittendrigh and Daan, 1976b;Zlomanczuk et al., 1991;Mason, 1991;de la Iglesia et al., 2000;de la Iglesia et al., 2003;Ohta et al., 2005). Animals may also become arrhythmic in their behavior, meaning that the animal is active and inactive irregularly throughout the 24 h day and no circadian rhythm can be observed (Pittendrigh and Daan, 1976b;Mason, 1991;Ohta et al., 2005).

Pittendrigh and Daan (1976b) developed a model for splitting that comprises two mutually coupled oscillators, an evening (E) and a morning (M) oscillator. If splitting occurs, both oscillators become 180 degrees out of phase with each other (Daan and Berde, 1978). When research progressed, splitting was shown to result from the two suprachiasmatic nuclei getting 180 degrees out of phase (de la Iglesia et al., 2000;Herzog and Schwartz, 2002;Ohta et al., 2005). However, the left and right SCN were found not to be the evening and morning component which were envisioned by Pittendrigh and Daan (Herzog and Schwartz, 2002).

A split rhythm was also found when rats were put in an extremely short light-dark regime of 22 hours (de la Iglesia et al., 2004). This is called forced desynchronization. The gene expression in the ventral part of the SCN corresponded to the 22 hour light-dark schedule, while the gene expression in the dorsal SCN was free-running with a rhythm longer than 24 hours (de la Iglesia et al., 2004). Also in this example, two parts of the SCN are desynchronized in phase.

When animals are exposed to high intensity light, they will become arrhythmic in their behavior as well as in their electrical activity (Zlomanczuk et al., 1991). Arrhythmicity in the SCN was found not to be present at the cell-level. The Per1 expression in the neurons was still rhythmic, but the electrical activity patterns of the single neurons were desynchronized and scattered over the 24 h day (Ohta et al., 2005). Total asynchrony between the SCN neurons do not necessarily stop rhythmicity in

peripheral oscillators, but can lead to decoupling of the peripheral oscillators with the SCN (Granados-Fuentes et al., 2004).

Aschoff (1960) found that the endogenous rhythms of mammals in constant light conditions varied under different light intensities. In LL, with increasing light intensity, light-active animals increase their spontaneous frequency, which means that the endogenous rhythm becomes shorter, while dark-active animals decrease their endogenous frequency. Aschoff explained this 'rule' by introducing a parametric model of light intensity. As the light intensity becomes higher, the clock runs faster (for day-active animals).

It appears that this is partly true for cells. The light responsive cells in the SCN have a threshold value to respond to light, below which they do not, or only negligibly, respond to the light input. Above this threshold value, the reaction of the cell increases or decreases monotonically with light intensity (Meijer et al., 1986). The threshold values are reached during dusk and dawn transitions (Meijer et al., 1986). However, the beginning of a light exposure period contributes more to an overall change in discharge activity than later portions of the light period (Meijer et al., 1992). This indicates that light pulses have a more profound effect on phase changes in the SCN than light intensity.

2.7 Intercellular communication: coupling between neurons

In the previous discussion on photoperiod it was shown that differences in the encoding for day length in the SCN may be explained by a change in the phase distribution between the neuronal activity patterns. For long days, the neurons are more widely dispersed in their timing of activation than in short days.

Jet lag and constant light both lead to desynchrony between populations of neurons in the SCN. Constant light conditions can lead to asynchrony or to a desynchronization between the left and right SCN. Jet lag causes a temporal desynchronization between the dorsal and ventral SCN, but the dorsal and ventral SCN resynchronize after a few days. The question arises which mechanisms in the SCN may explain these phenomena.

For proper functioning of the SCN, synchronization and phase differences between neurons and subpopulations of neurons are important mechanisms. Without synchronization stable rhythms will not occur, and phase differences provide plasticity to the biological clock. Synchronization can only occur when neurons or neuronal subpopulations can interact. Neurons and subpopulations of neurons must be able to communicate to each other about their phases. There is little known about how the phase distribution information contributes to a functioning circadian clock. Also the underlying mechanisms of synchronization are unresolved. How do the neurons transmit phase information to each other? Are the same mechanisms involved in day length encoding, in constant light, and in jet lag?

It is known that the main Zeitgeber for the SCN is the daily light-dark cycle. The photic information is a direct input to the SCN from the retinal hypothalamic tract (RHT). The retinal ganglion cells of the RHT appear to utilize the neuropeptide pituitary adenylyl cyclase-activating peptide (PACAP) and glutamate to communicate with the SCN (Hofman, 2004). The ventral SCN holds most of the neurons that receive retinal input from these cells. These SCN neurons express γ-amino butyric acid (GABA) and, often, vasoactive intestinal polypeptide (VIP) and the peptide histidine isoleucine (PHI) (Colwell et al., 2003;Harmar et al., 2002). GABA and VIP are the most likely candidates that can synchronize neurons or neuronal subpopulations.

2.7.1 GABA

GABA is produced by most of the neurons present in the SCN (Moore, et al, 2002). Jet lag studies show that GABA plays an important role in the synchronization between ventral and dorsal SCN (Albus et al., 2005). Albus et al. show that, in the rat, after a phase delay, $GABA_A$ is used to synchronize the dorsal and ventral SCN. In control slices, bimodal peaks in electrical activity are observed in both the ventral and the dorsal part of the SCN, which appear to be caused by one endogenous peak and one peak that was imposed by the other part of the SCN. Using the $GABA_A$ receptor blocker bicuculline the imposed peak in both regions disappears, leaving only the endogenous peak. This indicates that $GABA_A$ communicates the

phase of the endogenous ventral peak to the dorsal SCN, and vice versa, the endogenous dorsal peak to the ventral SCN.

Furthermore Albus et al. (2005) show that $GABA_A$ works differently in the dorsal and ventral SCN. In the ventral SCN endogenous GABA has inhibitory effects, while in the dorsal SCN it elicits excitatory responses (Albus et al., 2005). This dual role of GABA was reported before in earlier studies (Wagner et al., 1997;Wagner et al., 2001;De Jeu and Pennartz, 2002) but also contested in other studies (Gribkoff et al., 1999;Gribkoff et al., 2003). In all these studies however, it was not clear where in the SCN the measurements were performed. The finding that GABA acts differently in dorsal en ventral SCN might be a solution to this debate.

For single cell recordings, Liu and Reppert (Liu and Reppert, 2000) reported an inhibition of neuronal firing when GABA was added to the culture media. The inhibition occurred at all phases of the circadian period. However, the GABA application also elicited phase shifts (Liu and Reppert, 2000). The direction and magnitude of these phase shifts was depending on the circadian phase of treatment. Liu and Reppert (Liu and Reppert, 2000) found that only GABA acting through A-type receptors can induce phase shifts. The inhibition was mediated both through the $GABA_A$ and $GABA_B$ receptor. Liu and Reppert (2000) also succeeded to synchronize two clock cells in the same culture with opposite phase angles by applying daily GABA pulses.

Recently, Choi et al. (2008) found that GABA-expressing neurons can switch from GABA-mediated inhibition to GABA-mediated excitation, due to the expression of Na^+-K^+-$2Cl^-$ Cotransporter isoform1 (NKCC1). NKCC1 is expressing itself more in the dorsal SCN, and predominantly during the night. This indicates that GABA-mediated excitation will mainly be present during the night in the dorsal SCN.

In conclusion, there is strong evidence that GABA plays an important role in the synchronization between the dorsal and ventral SCN. GABA thus might play a role in the interregional communication of phase information between populations of neurons.

2.7.2 VIP

There are many clues that VIP may play a role in intercellular synchronization, rather than in interregional synchronization. VIP plays two roles in the SCN. Firstly, it sustains circadian rhythms of single cells. Secondly, it synchronizes single cells to one another (Welsh, 2007).

VIP signals through VPAC2 receptors, as does PACAP (Harmar et al., 2002). It has been shown that both VPAC2 receptor deficient mice (vipr2-/-) (Harmar et al., 2002) as well as VIP deficient mice (vip-/-) (Colwell et al., 2003) show weakened locomotor activity rhythms.

Harmar et al. (2002) showed that VPAC2 receptor deficient mice show only weak locomotor activity rhythms and that these mice do not actually entrain to a light-dark regime; they only show masking. This became apparent by the immediate shift of the locomotor activity rhythm in vipr2-/- mice after a phase advance or delay, whereas wild-type mice needed several days to adjust to the new regime. Also dark pulses during the day caused an increment of activity in the VPAC2 receptor deficient mice, while wild-type mice barely reacted to these pulses (Harmar et al., 2002). Finally, Harmar et al also showed that expression of clock genes (mPer1, mPer2, mCry1, mBmal1) was dramatically reduced in VPAC2 deficient mice as compared to wild-types.

It is concluded that the VPAC2 receptor is essential for the expression of robust circadian rhythms of behaviour and that the predominant factor determining the pattern of wheel-running activity in vipr2-/- mice is masking by light. The behavioural phenotype of vipr2-/- mice is associated with a lack of coordinated clock gene expression in the SCN (Harmar et al., 2002). This suggests that the VPAC2 receptor is critical for the generation and/or maintenance of rhythmic activity in the SCN (Harmar et al., 2002).

Colwell et al. (2003) developed VIP/PHI deficient mice. These mice show similar characteristics as the VPAC2 deficient mice from Harmar et al. (2002): weak rhythmicity, masking effects to a light-dark cycle and no entrainment to the light-dark regime. The vip-/- mice also show an expanded duration of their activity period (Colwell et al., 2003). Furthermore, when treated with a skeleton photoperiod with two 1-hour light pulses per 24-hour

cycle, the vip-/- mice exhibited a split rhythm of two activity periods instead of one (Colwell et al., 2003).

It appears that loss of the VPAC2 receptor is slightly more severe than loss of VIP/PHI. This indicates that many of the symptoms caused by a deficient VPAC2 receptor are due to a loss of VIP/PHI. However, other ligands, such as PACAP, also act on the VPAC2 receptor. In VIP/PHI deficient mice, PACAP can still work on the VPAC2 receptor, which may cause the less severe deficiencies in VIP/PHI deficient mice (Welsh, 2007;Colwell et al., 2003).

VIP/PHI deficient mice show masking effects to a light-dark regime, and when released in constant darkness the actual period to which they are entrained appears to have an extremely positive phase angle, as they start being active ~8 hours before lights off in the prior light-dark schedule (Colwell et al., 2003). This phenomenon is also found in the VPAC2 deficient mice and is a strong indication that VIP is required to synchronize the SCN to the external light-dark schedule (Colwell et al., 2003). Colwell et al. (2003) conclude that the function of VIP and the VPAC2 receptor can be explained in two, possibly complementary ways. First, VIP and the VPAC2 receptor may be required for the basic molecular oscillation in certain cells. Another possible explanation is that VIP and the VPAC2 receptor are directly involved in the communication between cell populations in the SCN (Colwell et al., 2003).

Aton et al. (2005) examined just these two possible functions of VIP by examining behavioral recordings and firing rates of individual neurons from vip-/-, vipr2-/- and wild-type mice. Harmar et al. (2002) and Colwell et al. (2003) used different breeds of mice for their knock-outs. Aton et al. (2005) therefore repeated their experiments in mice with the same genetic background. Compared to wild-type mice, the free-running rhythms of both vip-/- and the vipr2-/- mice were equally low and about the same percentage of mice expressed multiple periods. This confirmed that the rhythms in vip-/- and vipr2-/- mice both expressed weak circadian rhythms which are less synchronized than wild-type mice, but no differences were found between both knock-out mice (Aton et al., 2005).

Furthermore, in wild-type mice, about 70 % of the SCN neurons show circadian rhythmicity, while for both mutant types this was reduced to only 30 %. It appeared that a large proportion of neurons lost rhythmicity in the mutant mice (Aton et al., 2005). The single cell rhythms observed in the VIP and VPAC2 deficient mice are decreased in amplitude. This also indicates that intercellular signalling in the SCN, which regulates cycle-to-cycle stability of the circadian period, is decreased. Aton et al. (2005) measured that in high-density dispersals the period distribution between the SCN neurons is higher than in wild-type mice, which indicates a loss of synchrony. When a VPAC2-specific agonist was applied on a daily basis to vip-/- mice, the number of rhythmic neurons was restored to the level of wild-type mice (Aton et al., 2005), which further indicates that the VPAC2 receptor suffices for maintaining rhythmicity and synchrony between SCN neurons. Thus, Aton et al. (2005) conclude that VIP signalling through the VPAC2 receptor is promoting circadian rhythmicity in a subset of SCN neurons and it maintains synchrony between intrinsically rhythmic neurons.

In cell cultures, neurons can not synchronize their rhythms as well as in brain slice preparations in which the SCN network is preserved (Brown et al., 2007;Welsh, 2007). Maywood et al. (2006) show strong evidence for a role of VPAC2 receptors in SCN synchrony (Welsh, 2007). Maywood et al. (2006) used bioluminescence profiles to assess Per1 gene expression in the SCN and found that, compared to wild-type mice, the circadian rhythm in vipr2-/- slices was low in amplitude and also unstable, for it damped rapidly. These weak rhythms in gene expression may provide an explanation for the weak behavioural rhythms of these mutant mice (Maywood et al., 2006).

In wild-type brain slices, most Per1-expressing cells were circadian and their activity patterns were synchronized to a 4-5 hour interval. In vipr2-/- slices fewer rhythmic cells could be detected and the ones that were rhythmic were desynchronized (Maywood et al., 2006). Thus, vipr2-/- mice have a weakened rhythm in Per1 expression and the cells were desynchronized in their activity. These results confirm the results found by Harmar et al. (2002).

By depolarizing with K^+ or treatment with GRP, vipr2-/- mice can temporally get higher luminescence levels indicating that more cells become

rhythmic in Per1 gene expression and also synchronize more closely. When the treatment is stopped, the effect diminishes, because of the absence of VIPergic signals (Maywood et al., 2006). Thus, VIP appears to be essential for maintaining intrinsic synchrony of molecular rhythms in the intact SCN.

Brown et al. (2007) measured extracellular multiunit electrical activity patterns from SCN slices. The vip-/- mice showed a very weak electrical activity rhythm with variable periodicity and many peaks that were mutually out of phase. In accordance to other studies, the single cell electrical activity recordings showed that less cells in the vip-/- mice were rhythmic compared to wild-type (Brown et al., 2007). Furthermore, the vip-/- neurons showed decreased amplitude, the period was more irregular and the neurons were not clustered in phase for their peak times (Brown et al., 2007). This means that neurons in vip-/- mice are less capable of synchronizing their activity patterns to environmental light-dark schedules and to each other.

For adult mice, Brown et al. (2007) showed that the vipr2-/- mice show more severe disruptions in circadian rhythmicity than the vip-/- mice. This provides additional evidence that the VPAC2 receptor potentially carries out actions that do not involve VIP, as stated before by Colwell et al. (2003). PACAP may be a possible candidate to act on the VPAC2 receptor. Although SCN neurons do not produce PACAP, in adult brain slices RHT terminals that can produce PACAP are present in the SCN. These can be a source to activate VPAC2 receptors (Brown et al., 2007). Aton et al. (2005) used young mice in which the RHT terminals are not yet present in the SCN. This might explain why they did not find differences between vip-/- and vipr2-/- mice.

To conclude, VIP is important in coordinating the rhythmicity in the SCN, both by synchronizing intrinsically rhythmic neurons, as well as promoting circadian rhythmicity in other neurons.

2.7.3 Gap junctions

Besides chemical coupling mechanisms, electrical coupling mechanisms, or gap junctions, are often observed as mechanisms of communication between neurons in other parts of the brain. Long et al. (2005) showed that in the SCN about 26 % of the neurons are electrically coupled. They stimulated

one of a pair of neurons and measured electrical activity in both neurons. Compared to other brain areas, the percentage of electrically coupled neurons in the SCN is relatively low. Long et al. (2005) propose that electrical coupling in the SCN only occurs between neurons in specific subpopulations, in order to strengthen the output of these subpopulations. If this is the case, then electrical synapses in the SCN may form distinct networks of coupled neurons.

Long et al. (2005) show that gap junction protein connexin 36 (Cx36) seems to be the primary (if not exclusive) constituent of the electrical synapses in the SCN, because all electrical coupling was absent in Cx36-knockout mice but not in wild-type. The locomotor activity patterns of Cx36-knockout mice showed deficits in circadian behavior, especially in constant dark conditions, as well as a delayed onset of activity in the first DD cycle and a sustained reduction in circadian amplitude, indicating that light is entraining and amplifying the relatively weak circadian rhythms (Long et al., 2005). Therefore, electrical synapses, in the form of gap junctions, may play a role in the SCN for intercellular communication in order to maintain circadian rhythmicity.

Unlike chemical coupling, electrical coupling is bidirectional and symmetrical. It was observed in both dorsal and ventral SCN and the coupling strength appeared to be higher during the light phase of the light-dark cycle (Long et al., 2005). This means that the electrical coupling itself may be changing throughout the daily light-dark cycle (Colwell, 2005). This may indicate that gap-junctions actively regulate the communication between neurons depending on the time of the day (Colwell, 2005).

2.7.4 Coupling in the SCN

Summarizing the discussion, GABA seems to have an important share in the interregional communication of phases between the dorsal and ventral SCN. VIP and gap junctions might be working between neurons in a region, each in their own way. VIP might synchronize single cells within the dorsal and ventral region. GABA might synchronize populations of neurons to each other, such as the dorsal and ventral SCN. Gap junctions may strengthen certain groups of neurons in their coordinated output, which may vary in

strength throughout the daily cycle. Because of the low percentage of gap junctions in the SCN, the gap junctions may work for very specific groups of neurons.

2.8 Computer models and computer simulations of the clock

Over the years, computer models were constructed for the biological clock and computer simulations were performed to gain more information about the clock. The following section provides an overview of the circadian clock models that have emerged over time.

Around 1960, the Cold Spring Harbor Symposium on Quantitative Biology on Biological Clocks was held. At that time, not much was known about the circadian system. The location of the clock in mammals was not identified. Therefore one could only study behavioural data and temperature data. The studies revealed that there had to be an endogenous oscillatory mechanism in organisms, and that this mechanism could be perturbed by the influence of a light-dark schedule.

During the aforementioned symposium, there was general agreement on the endogenous nature of circadian rhythms (all except for the Brown lab agreed on this at that time). This endogenous clock was often represented as a single oscillator (Klotter, 1960a;Klotter, 1960b;Kalmus and Wigglesworth, 1960). These single oscillator models were fit to the behavioural experimental data available at that time. Limit cycle oscillators were used by Kalmus and Wiggelsworth to describe the pacemaker (its 'phase portrait'), where the system was in a 'phase' (referred to as θ) specified by two variables *x* and *y*, which vary over time. The dynamic system of the clock is since then often described by limit cycle oscillators. An introduction into these limit cycle oscillators seems therefore appropriate.

2.8.1 Interlude: Limit cycle oscillators

A system is called dynamic if it transfers from one state to another over time. Each state of the system can be described in terms of *N* variables, the so-called *state variables*. The number of variables involved in defining a state is the number of *dimensions* of the dynamic system (Pikovsky et al., 2001). If

the values of the state variables repeat themselves periodically, the system shows oscillatory behavior.

In order to gain a better understanding of the mathematical principles involved in oscillatory systems, a two-dimensional model system for the relation between the populations of a prey and a predator will be discussed. The two state variables in this system are the populations of both species. In an idealized situation, if the population of the prey is growing, then the population of the predator will follow. At a certain time, the numbers of predators become so high that the population of prey starts to decline. This, in its turn, causes the predator population to decrease. An example is presented in the figure 2.5 A. If we plot both populations in one graph on the two axes, we get a picture that resembles the one in figure 2.5 B.

Note that in figure 2.5 B the two state variables involved in the system are used for the axes. The curves in this state-variable-plot are closed, meaning that the system returns to a previous state every time after a period *T*. The closed curve is called a trajectory. This closed trajectory is called a *limit cycle* (Pavlidis, 1978b).

The equations belonging to the predator-prey system are (see Goel et al., 1971)

$$\frac{dx}{dt} = x(\alpha - \beta y) \tag{1}$$

$$\frac{dy}{dt} = -y(\gamma - \delta x), \tag{2}$$

where

- y is the number of some predator (for example, wolves);
- x is the number of its prey (for example, rabbits);
- t represents the time;
- dy/dt and dx/dt represents the growth of the two populations against time;
- β and δ are parameters representing the interaction of the two species; and
- α and γ are parameters representing the separate evolution of the two species.

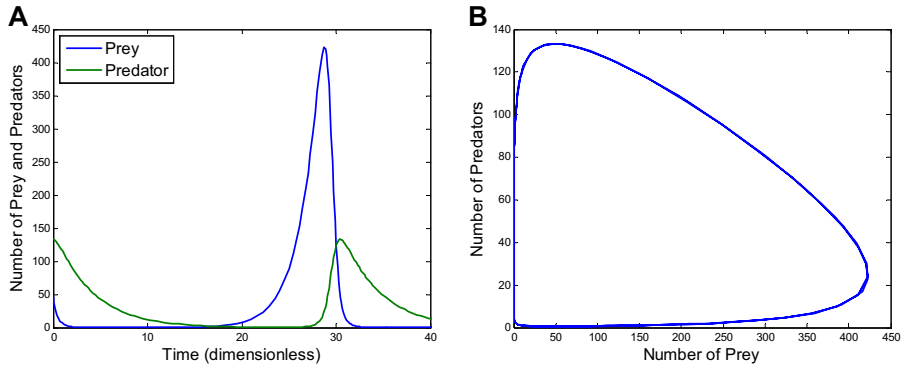

Figure 2.5 Predator – prey models. The left figure shows the population size over time. In the right figure the population sizes are plotted against each other.

Or written in a more general way

$$\frac{dx}{dt} = f(x, y)x \tag{3}$$

$$\frac{dy}{dt} = g(x, y)y. \tag{4}$$

The state of the system at a certain moment is defined by the values of the different state variables. In a limit cycle oscillator, the states of the system will return after its period *T*. It will always return to that state. We can also define the state of such a system to its *phase*, which is the angle on the cycle with respect to some initial phase (which is defined as 0). If the phase is at timepoint *T*, or 360°, the system is back at its original phase. Thus, at some timepoint t_0, the system is in a certain phase of the cycle, and after one period *T*, so at timepoint t_0+T it returns to the same phase (Winfree, 2000).

The different state variables are coordinates in the phase space (see figure 2.6 B), and its plot is called a phase portrait and the point on the limit cycle constructed by the values of the state variables is called a phase point (Pikovsky et al., 2001).

The phase as described in limit cycle models of the biological clock can be understood as subjective circadian time (CT) of the clock (Pavlidis, 1978b). Note that this phase is not one of the state variables involved in the limit cycle characteristics of the clock. What the state variables are that

describe the limit cycle behavior of the clock is not known (Kawato et al., 1982).

A special property of the limit cycle is that the original rhythm is restored when it is perturbed, just like the endogenous rhythm of the clock. Small deviations from the limit cycle trajectory tend to return to the curve after some time (Pikovsky et al., 2001).

One example of a limit cycle oscillator is the Van der Pol oscillator. Van der Pol equations describe an oscillator that has nonlinear damping, and can be used to describe the oscillations of the biological clock in a mathematical manner (Wever, 1972;Kronauer et al., 1982;Kronauer, 1990). Within certain parameter boundaries, the oscillator will become attracted to, or entrained to, the limit cycle, which ensures stable oscillations (Kalmus and Wigglesworth, 1960).

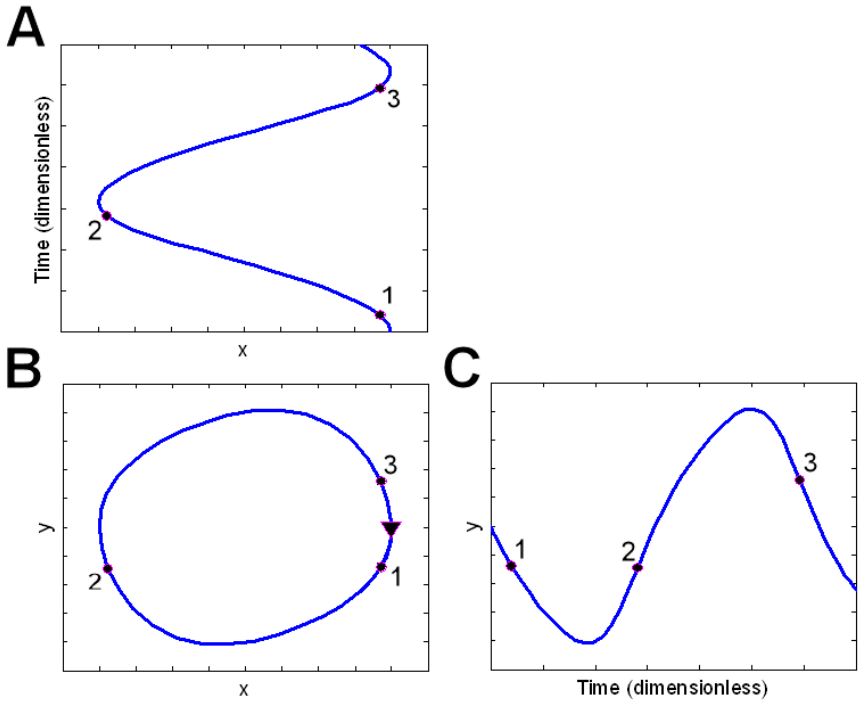

Figure 2.6 Limit cycle oscillators and their phase portrait. The two oscillating state variables *x* and *y* are oscillating in time (A and C). When the variables are plotted against each other in the phase space, the phase portrait of these two states is shown in B, where *x* is plotted on the x-axes, and *y* on the y-axes. This is a periodic function. When starting one period at the arrow, first point 1 is encountered, then point 2, and finally point 3.

2.8.2 Two-oscillator models

Before and during the Cold Spring Harbor Symposium, the endogenous circadian clock was represented as one single oscillator. However, certain behavioural results could not fully be explained by one oscillator.

In 1958 a two-oscillator model was proposed that could explain transient behaviour in phase resetting of the *Drosophila*. The so-called A oscillator is a pacemaking, self-sustaining, temperature-compensated system, which is the ultimate clock and is sensitive to light. This oscillator is supposed to shift immediately after light input. The second oscillator (called B) is coupled to and driven by the first oscillator (A) and is supposed to be light-insensitive but temperature-sensitive. Oscillator B drives the behavioural pattern. Resetting light input directly drives the A oscillator. The B oscillator takes several cycles to be re-entrained by the A oscillator, explaining the transients in behaviour (Pittendrigh et al., 1958;Pittendrigh and Bruce, 1959;Pittendrigh, 1960).

In 1976, in their famous series of articles, Pittendrigh and Daan (1976b) suggested another two-oscillator model in order to explain the phenomenon of splitting. Splitting involves a 24-h behavioural rhythm which consists of two periods of rest and of activity. The phenomenon of splitting was mainly found in hamsters. In this model the clock consists of a morning (M) and evening (E) oscillator. Daan and Berde (1978) described a mathematical system based on the E and M oscillator. They used two limit cycle oscillators, one for the morning component and one for the evening component. Each oscillator had its own endogenous period, but both oscillators were able to influence the other by resetting each others phase by a small amount on the basis of a phase resetting curve.

According to Kawato and Suzuki continuous resetting between oscillators is more plausible than resetting according to a phase resetting curve (Kawato and Suzuki, 1980). For this reason they used two identical limit cycle oscillators that are coupled continuously. If one oscillator is in rest, it does not affect the other oscillator and the coupling is symmetrical (Kawato and Suzuki, 1980). Splitting can be explained by the two steady states in which the oscillators can be synchronized: either they are *in-phase*, having a phase angle of 0°, or they are in *opposite* phase, which means that their phase is

180° separated (Kawato and Suzuki, 1980). This two-oscillator model could also describe transient phase resetting (Kawato, 1981).

Next to the two-oscillator models of Pittendrigh, Aschoff and Wever also studied two-oscillator systems that represented the clock. From the first extensive bunker experiments with human subjects (Aschoff and Wever, 1962;Aschoff, 1965a), Aschoff and Wever found that the rhythms in core body temperature and the sleep-wake cycles could diverge completely, a state they named 'internal desynchrony' (Aschoff, 1965a;Wever, 1985;Wever, 1989).

Wever modeled the 'clock' as a series of differential equations based on the Van der Pol equations (Wever, 1972). For all general clock properties, including entrainment in different light conditions according to Aschoff's rule, this model is sufficient (Aschoff, 1960;Wever, 1962;Wever, 1972). However, for special properties, like internal desynchrony, a second oscillator that is controlled by the first oscillator needs to be added to the model (Wever, 1989;Wever, 1962).

Kronauer designed a model in 1982 that was based on sleep research on humans (Kronauer et al., 1982). Humans also seemed to have two oscillatory processes: the sleep-wake process and a temperature oscillator. Kronauer modeled these by two Van der Pol oscillators. Also in this system of differential equations the coupling is continuous, as in the model of Kawato and Suzuki (Kawato and Suzuki, 1980) and unlike the model of Wever. In Wever's model, the second oscillator was controlled by the first oscillator but did not feed back to the first oscillator (Wever, 1962;Wever, 1989). By decomposing the sleep-wake process oscillator into two oscillators, one for a wake cycle, which determines the onset of sleep, and one oscillator for the sleep cycle, determining the onset of awakening, Kawato et al (1982) created a three oscillator model to describe the problem of internal desynchrony.

Note that a two-oscillator model can be described by one limit cycle oscillator. However, the dimensionality of the oscillator would then increase and the mathematics would become difficult to understand, and would not contribute to the understanding of the circadian clock mechanism (Kawato, 1981). Using two oscillators provides a better understanding of the mechanisms that may govern the clock.

The two-oscillator models could thus explain splitting, transient behaviour, after-effects and Aschoff's rule. In the 1980s the sleep homeostasis 2-process model was introduced. By that time there was general consensus that the suprachiasmatic nuclei contained the mammalian circadian clock. When the electrical activity of the SCN could be measured (Groos and Hendriks, 1982) the research focused on the activity rhythms of the SCN itself, instead of the driven rhythms such as the behavioural or temperature rhythms.

With the process S model for the sleep-wake cycle in place and the available data of the SCN, Kronauer updated his earlier model in 1990. He created a model based on two Van der Pol oscillators and added the forcing effects of light to this model (Kronauer, 1990). This was fit to experimentally obtained temperature data from human subjects.

The model was as follows

$$\frac{24}{2\pi}\frac{dx}{dt} = y + \varepsilon(x - \frac{4}{3}x^3) + B \tag{5}$$

$$\frac{24}{2\pi}\frac{dy}{dt} = -(\frac{24}{\tau})^2 x + By \tag{6}$$

$$B = (1 - mx)CI^{\frac{1}{3}}, \tag{7}$$

where

- x and y are the state variables of the oscillator,
- τ denotes the endogenous period of the oscillator,
- ε is the stiffness of the oscillator,
- B is the brightness; which is the influence of light on the oscillator,
- I is the light intensity in lux,
- m the modulation index weighting the magnitude of the feedback of the oscillator,
- C a constant.

This model was able to predict experimentally obtained data (Klerman et al., 1996), however it did not adhere to experimental data obtained in bright or dim light conditions and after brief light stimuli. Therefore the Kronauer model was refined (see figure 2.7). The light input was updated to contain a

new pre-process, called L, which processes the light intensity *I* and produces a drive $\hat{B}$ that serves as the light input into the circadian system, which is called process P. Process P contains a stimulus modulator (*S.M.*) and the circadian oscillator (~). The stimulus modulator (*S.M*) does not receive direct light input, but receives the drive $\hat{B}$. Depending on the phase of the oscillator, the stimulus modulator (*S.M.*) produces a modulated drive *B*, which can adjust the phase and amplitude of the oscillator (~). The process that modulates the light input in the previous model was only depending on the state variable *x*. In the new model this process is replaced by the stimulus modulator (*S.M.*) and this process is now dependent on both state variables *x* and *y* (Kronauer et al., 1999;Jewett et al., 1999).

Interesting new viewpoints were provided by Achermann and Kunz (Achermann and Kunz, 1999;Kunz and Achermann, 2003). Where Kronauer and his team used the Van der Pol oscillator in their model as a single oscillatory unit describing the SCN as a whole, Achermann and Kunz use this same oscillator model to describe only one oscillatory neuron in the SCN. They used the model of Kronauer (Kronauer, 1990) and added a coupling term to each oscillatory unit, so that the oscillators can 'communicate' about their phase and amplitude with other oscillators in their neighborhood, through the use of local coupling with either 4, 8 or 20 surrounding cells. They placed 10,000 of these oscillators in a regular grid of 100 by 100 units. The global output of the SCN as a whole is given as the arithmetic average of the values of all oscillators (Achermann and Kunz, 1999). This was an attempt to create a model on the basis of a network of oscillators. However, the coupling mechanisms between the units were not very realistic, because they were based on the mathematical notion of neighborhood coupling.

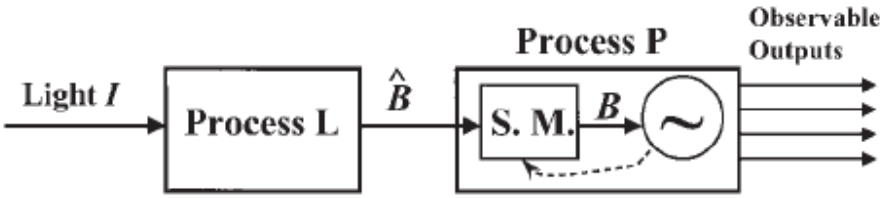

Figure 2.7 Updated model for circadian rhythms and light influence. The light input enters the clock through a pre-process, called L, which processes the light intensity *I* and produces a drive $\hat{B}$ that serves as the light input into the circadian system, which is called process P. Process P contains a stimulus modulator (*S.M.*) and the circadian oscillator (~). The stimulus modulator (*S.M*) does not receive direct light input, but receives the drive $\hat{B}$. Depending on the phase of the oscillator, the stimulus modulator (*S.M.*) produces a modulated drive *B*, which can adjust the phase and amplitude of the oscillator (~). (Reprinted from Journal of Biological Rhythms 14, Jewett et al, 1999).

2.8.3 Molecular models

It has become clear that the circadian system is a heterogeneous system containing endogenously oscillating pacemaker cells. This insight affected the construction of the models. Some models were developed to describe primarily the interaction between the neurons to describe the SCN network output. The second line of research focused on modeling the endogenous pacemaker cell, and the generation of circadian rhythms itself.

The Goldbeter group developed an impressive number of molecular models for circadian pacemaker cells of different species (Goldbeter, 1995;Leloup and Goldbeter, 1998;Leloup et al., 1999;Leloup and Goldbeter, 2000;Leloup and Goldbeter, 2001;Leloup and Goldbeter, 2003;Leloup and Goldbeter, 2004). The models described the circadian expression of genes and their protein products. These models were based on the model first proposed by Goodwin, where a protein that represses the transcription of its own gene is able to produce sustained oscillations in the levels of protein and mRNA (Goodwin, 1965;Griffith, 1968). Drescher was the first to use this model in the field of circadian rhythms to determine PRCs with respect to transient perturbations (Drescher et al., 1982).

The first model from the Goldbeter group described the rhythmic expression of Per in the Drosophila clock (Goldbeter, 1995). The period of

the rhythm arises because of the cascade of phosphorylation states the protein goes through, in which each step introduces a time delay.

The following kinetic equations describe this model (see figure 2.8)

$$\frac{dM}{dt} = v_s \frac{K_I^n}{K_I^n + P_N^n} - v_m \frac{M}{K_m + M} \tag{8}$$

$$\frac{dP_0}{dt} = k_s M - V_1 \frac{P_0}{K_1 + P_0} + V_2 \frac{P_1}{K_2 + P_1} \tag{9}$$

$$\frac{dP_1}{dt} = V_1 \frac{P_0}{K_1 + P_0} - V_2 \frac{P_1}{K_2 + P_1} - V_3 \frac{P_1}{K_3 + P_1} - V_4 \frac{P_2}{K_4 + P_2} \tag{10}$$

$$\frac{dP_2}{dt} = V_3 \frac{P_1}{K_3 + P_1} - V_4 \frac{P_2}{K_4 + P_2} - k_1 P_2 + k_2 P_N - v_d \frac{P_2}{K_d + P_2} \tag{11}$$

$$\frac{dP_N}{dt} = k_1 P_2 - k_2 P_N \tag{12}$$

The total quantity of PER protein is given by

$$P_t = P_0 + P_1 + P_2 + P_N , \tag{13}$$

where

- M is the concentration of *per* mRNA
- P_0 is the concentration of unphosphorilated PER protein
- P_1 is the concentration of monophosphorilated PER protein
- P_2 is the concentration of biphosphorilated (or fully phosphorilated) PER protein
- P_N is the concentration of PER protein in the nucleus
- P_t is the total quantity of PER protein
- v_s is the maximum rate of accumulation of Per mRNA in the cytosol
- v_m is the maximum rate of cytosolic Per mRNA degradation
- v_d is the maximum rate of degradation of biphosphorylated PER protein
- $V_{1\text{-}4}$ denote the maximum rate of kinase and phosphotase in the reversible phosphorylation of P_0 into P_1 and P_1 into P_2.

- k_s is the first-order rate constant for the synthesis of the PER protein (P_0) from Per mRNA.
- k_1 is the first-order rate constant for the transport of fully phosphorylated PER protein (P_2) into the nucleus (P_N)
- k_2 is the first-order rate constant for the transport of nuclear PER protein (P_N) into the cytosol (P_2)
- K_m, K_d, and K_{1-4} are Michaelis constants
- K_I is the threshold constant for repression of *per* transcription by nuclear PER protein concentrations (P_N)

This model shows a rhythm in total PER protein (P_t) and in Per mRNA level (M). These two levels are treated as state variables for the limit cycle oscillator.

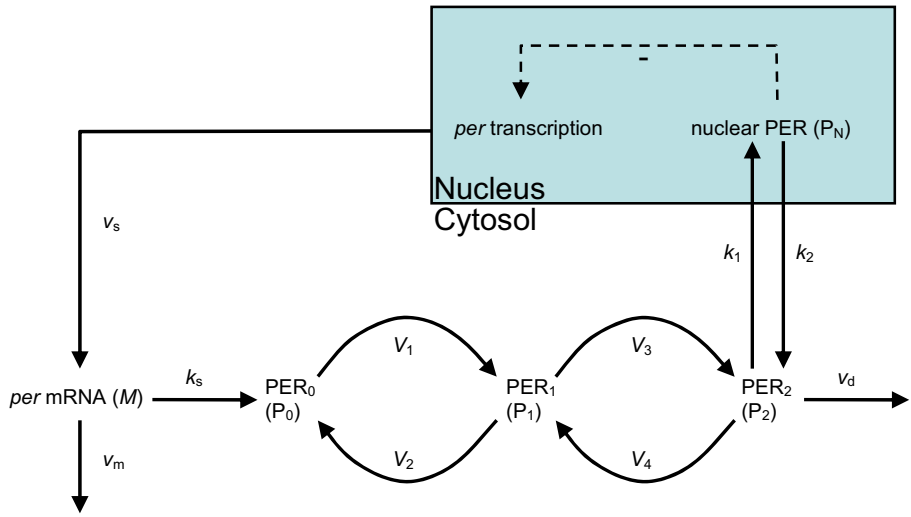

Figure 2.8 Molecular model for Per expression in the Drosophila clock. *per* mRNA (M) is synthsized in the nucleus and transfers to the cytosol, where it accumulates at a maximum rate v_s. In the cytosol, the *per* mRNA is degraded with maximum rate v_m and synthesized into PER protein with a maximum rate k_s. PER protein can be present in different phosphorylation states, P_0, P_1, and the fully phosphorylated form P_2. The maximum rate of phosphorylation and dephosphorylation between the phosphorylation states is characterized by the parameters V_i. P_2 is degraded at a maximum rate v_d and transported into the nucleus according to rate constant k_1. The transport of the nuclear, fully phosphorilated form of PER (P_N) into the cytosol is characterised by constant k_2. The nuclear PER (P_N) negatively feeds back on *per* transcription. (Reprinted from Figure 1 in Goldbeter, A., 1995. A model for circadian oscillations in the Drosophila period protein (PER). Proc. R. Soc. Lond. B 261, page 320, with kind permission of Royal Society Publishing)

In 1998, the Tim gene cycle was added to the model (Leloup and Goldbeter, 1998). Two interlocked loops can account for more complex behaviour as is observed from the circadian system, such as rhythm splitting and arrhythmicity. The equations that are used to describe this model are similar to the ones that describe the simpler version of this model, with only Per. The model is depicted in figure 2.9.

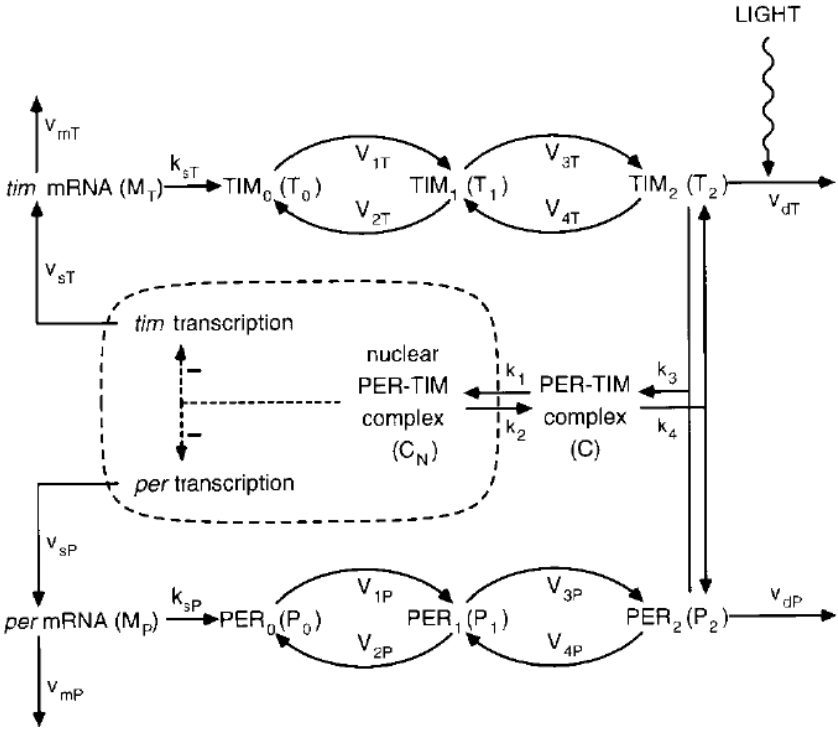

Figure 2.9 Addition of the Tim gene cycle describing the clock of *Drosophila* to the model described in figure 2.8 (Reprinted from Journal of Biological Rhythms 13, Leloup and Goldbeter, 1998).

Different modifications of the model were made, and the model was also designed for the Neurospora circadian clock (Leloup et al., 1999;Leloup and Goldbeter, 2000;Leloup and Goldbeter, 2001). When the clock genes for the mammalian clock were identified, the model was transferred to a mammalian circadian clock model, involving the Per, Cry, Bmal1, Clock, and Rev-Erbα genes (Leloup and Goldbeter, 2003;Leloup and Goldbeter, 2004). A similar molecular model for the mammalian clock was created by Forger and Peskin. In their model more effort was made to precisely mimic

the molecular components that play a role in the clock cell (Forger and Peskin, 2003;Forger and Peskin, 2004;Gallego et al., 2006).

2.8.4 Network models

In 1967, Winfree speculated on the clock being a network of coupled oscillators (Winfree, 1967). Interestingly, Daan and Berde also speculate on the nature of both oscillators, and suggested that each may consist of a multitude of tightly coupled oscillators (Daan and Berde, 1978).

Winfree was probably the first who proposed that certain clock properties were network properties instead of the properties of a single oscillator (Winfree, 1967). In order to investigate the rhythmical interaction of populations of periodic processes, he introduced the "generalized relaxation oscillator". This is a limit cycle oscillator which has weak coupling. The phase (φ) is always close to the phase of an entraining periodic stimulus (θ). The maximal difference from θ is ψ. Besides the influence of the Zeitgeber (θ), the oscillators themselves also influence each other's phase. The two state variables for the limit cycle oscillators are the Influence Function $X(\varphi)$, which represents "all effects by which an oscillator makes its presence and phase known to others", and the Sensitivity Function $Z(\varphi)$, which represents the "sensitivity to stimuli of some sort S with which we will perturb it". Using these two state variables, Winfree was able to simulate a population of oscillators, that had similar, but not necessarily the same, periods. These oscillators were attracting each other and became synchronized with a stable period as though it was one oscillator. With this intuition, Winfree was the first to suggest that the circadian clock consisted of a multitude of differently phased oscillators, that were synchronized in order to produce a unified output signal (Winfree, 1967).

A number of other studies followed this line of thought, where 'simple' oscillators were placed in a network and coupling was studied using the theoretical mathematical mechanisms of coupled oscillators (Pavlidis, 1971;Pavlidis, 1978a). The theory of coupled oscillators was discovered by the famous Dutch scientist Christiaan Huygens when looking at two pendulum clocks swinging in perfect synchrony, and is discussed in relation to circadian clocks by Strogatz and coworkers (Strogatz and Stewart,

1993;Mirollo and Strogatz, 1990;Strogatz, 2003). The study of coupled oscillators showed that a stable rhythm could arise from a population of less reliable oscillators (Enright, 1980a;Enright, 1980b).

The network models discussed use artificial oscillators that represent the neurons in the SCN. Furthermore, they use theoretical connection and synchronization schemes between the single units. The network models that follow gain realism. First, more realistic molecular models are introduced to represent the neurons in the network models. Second, the network topology of the SCN is taken into account in the coupling mechanisms of the SCN.

Bernard et al. (2007) have modeled SCN neurons in a similar fashion as the Goldbeter group. However, in their study, a coupling term was added to the equations of the oscillators, where the coupling was based on the concentration of one of the phosphorylation phases of the PER/CRY protein complex. The coupling between the oscillators was tested using three types of coupling: random coupling, nearest neighbor coupling and a coupling scheme based on the ventral-dorsal and left-right distinction of the SCN. Their main finding was that oscillators of which the rhythms would normally damp out, could remain rhythmic when they were coupled to other damped oscillators. The coupling between damped oscillators actually sustained the rhythmicity of the network (Bernard et al., 2007).

The work of Achermann and Kunz can also be seen as representatives of the models that add more realistic molecular models to the network models (Achermann and Kunz, 1999;Kunz and Achermann, 2003). However, the coupling schemes are still derived from mathematical constructs, and not so much from realistic coupling between SCN neurons. The models from Bernard et al and Achermann and Kunz use theoretical coupling patterns to describe the interaction between the single cells instead of more realistic coupling mechanisms between the neurons. Other models turned their focus on more realistic coupling schemes between the single cells.

Antle et al. (Antle et al., 2003;Antle et al., 2007) model the SCN as consisting of two regions, the ventral region and the dorsal region. The “gate cells”, situated in the ventral region of the SCN, synchronize the oscillator cells, which are situated in the dorsal SCN. An oscillator cell is modeled by

a polar parameterization of the Van der Pol equation, suggested by Forger and Kronauer (2002)

$$\frac{dr}{dt} = -\omega r \cos(\theta)^2[-1 + r^2 \cos(\theta)^2] \quad (14)$$

$$\frac{d\theta}{dt} = \omega[-1 + \varepsilon r^2 \cos(\theta)^3], \quad (15)$$

where

- the polar coordinate form uses an angle, θ, and a radius, r, to map a point's location relative to the origin
- ω is the frequency of the oscillator
- ε is the "stiffness" coefficient that determines how closely the oscillator mimics a cosine curve.

The gate cells in the ventral SCN reset an oscillator by pushing its phase closer to the average phase of the ensemble in a linear fashion. The resetting function has two parameters. The *slope* of the resetting function represents the strength of the resetting. A slope of 0 would bring every oscillator back to the gate point every time the gate fired; a slope of 1 would have no effect. The second parameter is the *point of intersection with null resetting function*, which determines what phases are advanced and what phases are delayed when the gate is triggered. Note that the oscillators themselves are not coupled in this model. Antle, et al. conclude that this model shows that gate cells, which are under the influence of light, can be important for creating synchronization between the oscillating cells in the SCN and different strengths of the gate cells can explain a decreased synchronization (Antle et al., 2003).

Gomes Cardoso, et al. defined a model that takes into account the biological realism in the coupling and especially in different SCN regions (Gomes Cardoso et al., 2009). The ventral region is an input region which sends output to the dorsal region, similar to the model of Antle, et al. (2003). The dorsal region is defined as a three-dimensional grid, which resembles the model of Achermann and Kunz (1999). In the dorsal region the neurons are connected to each other, in contrast to the Antle model, because in that model the dorsal neurons were not coupled to each other. The dorsal neurons

are able to modify each others frequencies because of this coupling (Gomes Cardoso et al., 2009).

Beersma et.al. (2008) extends the coupled oscillator studies by adding to the coupling mechanisms of the oscillators more realistic SCN neuronal properties, without trying to model precisely the cell itself. A cell can be actively firing or be silent. The exact mode of operation can be influenced by the light dark cycle and by other pacemaker cells. All cells are influencing all other cells. Using a simplified version of a single cell representation instead of a realistically feasible neuronal model, the authors are able to obtain SCN properties that arise purely from the network level (Beersma et al., 2008).

Many other models have been created in the field of circadian rhythms. This selection provides a sufficient overview of the different lines of research in modeling the biological clock. A number of modeling techniques have been discussed and different approaches have been mentioned. One can model one single neuron, or one can model the whole SCN. One can focus on realistic models of the neuron, or one can focus on coupling between neurons. Depending on the research question one approach is preferred over another.

2.9 Conclusions

The circadian clock is endogenously rhythmic which can be explained by a molecular feedback loop within neurons of the SCN. These endogenously rhythmic neurons are synchronized to each other to produce a rhythmic output pattern. Synchronization of the neurons can be explained by different coupling mechanisms between the neurons. The clock can be entrained to the environmental light-dark cycle and responds to seasonal differences in day length. Different functional regions can be distinguished in the SCN, which have a specific function in the entrainment of the clock. Within and between these regions, neurons may show different coupling mechanisms. To investigate the organization of the SCN and the coupling mechanisms between neuronal subpopulations, computer models and simulations can be employed to guide experiments and gain insight in possible working mechanisms.

Models are always based on reality. They are designed to provide answers to questions related to everyday life. However, it is not always necessary to create a model that captures as many aspects of reality as possible. Sometimes, a 'simple' model that connects to only one small property of reality is as good as any larger model that tries to explain many aspects of real life. An important criterion for the use of a model is that the model should be sufficient to address the research question at hand. Depending on the research question the complexity of the model and the simulations should be determined. The purpose of the simulations should be thoroughly considered. What is it that the simulation should do? Is it to show how a system works? Or should the simulation provide answers to certain specific questions?

One should always remember that simulations alone can not provide irrevocable evidence for the subject under investigation. However, if the boundaries are defined well, it is possible to find answers to a specific question. With the answers that are found, new questions arise. At least some of these questions need experimental testing. When a question is defined too strictly, it is not possible to answer questions that lie outside the model. If other questions need answers, such as the underlying mechanisms of certain phenomena, new models are required that are designed with close regard of the boundaries needed for that specific question.

If these constraints to modeling and simulation are taken into consideration, the results coming from simulations can provide interesting insights and can be very valuable to guide experiments and research. In the next chapters of this thesis, simple models are used to simulate attributes of the circadian timing system where the network organization of the SCN and the coupling between subpopulations of neurons is involved.