Photoperiodic encoding by the neuronal network of the suprachiasmatic nucleus

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CHAPTER 1

Introduction

Circadian Rhythms

The rotation of the earth around its axis causes differences in light intensity; temperature; food availability and wavelength composition of the light. Many organisms show rhythms in their behavior and physiology, which for long have been ascribed to environmental cycles. In 1926, Johnson concluded that there must be an internal clock. He based this conclusion on observations of mice, kept in constant conditions without environmental stimuli, which showed a sustained activity rhythm that deviated slightly from 24h (Johnson, 1926; Johnson, 1939). This is visible as a daily shift of the activity rhythm relative to the 24h cycle. Because of the deviation from 24h, it is unlikely that the rhythm is attributable to an external cycle. These rhythms in an organism of approximately 24h that continue to exist when environmental time cues are absent, are called circadian rhythms (circa = approximately; dies = day). The evolutionary advantage of circadian rhythms is that they allow an organism to anticipate to daily occurring events.

Advantage of a clock

For an organism that is able to anticipate the environmental changes, the fitness or reproductive success is thought to be enhanced. By studying unicellular cyanobacteria whose lifecycle is shorter than 24h but possess a circadian clock, the reproductive success can determined. The environmental time information, which exceeds the lifespan of these bacteria, is inherited by the offspring. When rhythmic and arrhythmic strains of these bacteria are exposed to a cyclic environment, the bacteria that express a circadian rhythm in metabolism are able to reproduce at higher rates than the arrhythmic strain, showing the adaptive significance of a clock that anticipates daily changes (Woelfle et al., 2004; Johnson et al., 2008). In higher organisms, it is much harder to find evidence for the functional evolutionary advantage of a circadian clock, although this concept has been studied in wild-caught animals. A number of animals received surgery that selectively abolished circadian rhythmicity. After surgery, the survival rate of these animals was monitored in their natural habitat. Despite a lack of a controlled environment, these studies presented compelling data that animals without circadian rhythms were more prone to predation than animals that did display a circadian rhythm (DeCoursey et al., 1997; DeCoursey and Krulas, 1998; DeCoursey et al., 2000). It is likely in the case of a nocturnal animal that it will have an advantage when it seeks refuge in its retreat before the sun rises, preventing predation by daytime hunters. Also, when an animal adjusts its timing so that it is foraging when a suitable food supply is more easily available, it can sustain a higher number of offspring, giving rise to a higher reproductive value.

Entrainment to environment

The research of circadian rhythms in mammals started with recordings of behavioral activity and many functions of the biological clock could already be investigated without knowledge of the actual location of such a clock, i.e. as a black box analysis. Although the internal clock continues to oscillate without environmental stimuli, it

is responsive to light and darkness. The internal free running rhythm, visible in the absence of time cues, can be phase advanced or phase delayed by presentation of a light pulse. The extend and direction of these phase shifts depend on the time of the light pulse. For instance, a nocturnal animal that is housed in darkness, responds to a light pulse at the beginning of the activity rhythm with a delay of its activity the following days. Light presentation at the end of its active period will phase advance the rhythm, in the resting phase of the animal, no phase shifts can be induced by light, even when the animal is awakened (DeCoursey, 1964; Daan and Pittendrigh, 1976a; Pittendrigh and Daan, 1976a; Takahashi et al., 1984). This time dependency of responses to light pulses can be represented in the form of a phase response curve (PRC). The PRC illustrates that the phase at which the light is presented, determines the response. Because of the time dependent responsiveness to light, animals will adjust their timing to the environment in natural conditions. Animals that have a free running period larger than 24h will return to their burrow too late and will therefore receive a phase advancing pulse in the morning. An animal with a period shorter than 24h will be entrained by the evening light at the start of the activity and will consequently delay its activity. This results in an adaptation of the internal clock to the environmental period. This process of adaptation to the external light dark cycles is called entrainment (Pittendrigh and Daan, 1976a).

Discovery of the suprachiasmatic nucleus

The clock is connected with the eyes

Because of the functional relation between the circadian oscillator and the environmental light conditions, a direct pathway was proposed between the optic pathway and the circadian clock. In an anatomical study the retino-hypothalamic tract (RHT) was identified (Moore and Lenn, 1972). The RHT is a tract that forms a direct connection from the retina to the hypothalamus. Because of this direct connection, the

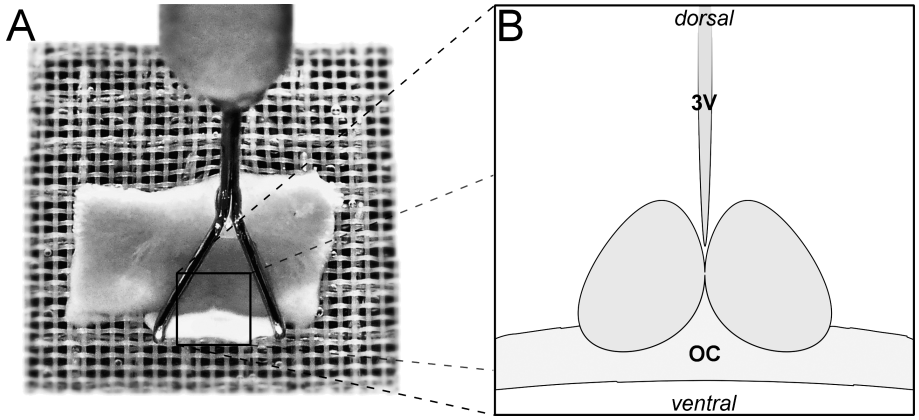

Figure 1.1

Coronal section containing the suprachiasmatic nucleus

(A) Photograph of a brain slice containing the SCN in an *in vitro* recording setup. The slice is stabilized by a fork and reaches from the dorsal part of the slice down to the ventral part, extending over the optic chiasm (OC, indicated in B). The area indicated by a black square is shown enlarged in B.
(B) Schematic drawing of a coronal hypothalamic section containing the SCN. On top of the optic chiasm (OC) the two suprachiasmatic nuclei are shown with the third ventricle (3V).

anterior part of the hypothalamus became a subject of great interest for a possible function in circadian rhythmicity.

Lesions along the optic tract were made and rhythmicity in wheel running, drinking and secretion of hormones, were recorded, to investigate at what point a lesion would induce a free-run of the circadian rhythm. It was hypothesized that, when no light-dark information from the eyes is transmitted to the clock, the clock will no longer synchronize to the environmental light dark cycles. As long as the lesions are upstream to the clock, this will be visible as a free-run of behavioral rhythms. When lesions of the optic nerve are performed downstream of the clock they do not affect entrainment. An experiment confirmed these hypothesis, lesions of the optic tract, downstream of the hypothalamus, rendering behaviorally blind animals, did not abolish entrainment capabilities (Moore and Klein, 1974; Klein and Moore, 1979).

Two research groups identified a paired structure just above the crossing of the optic nerve, the suprachiasmatic nucleus (SCN) as a possible location of the circadian oscillator at the same time (Moore

and Eichler, 1972; Stephan and Zucker, 1972). When the SCN was electrolytically lesioned, the rhythms in various output parameters were absent. This indicated that the SCN is critical for the generation of a circadian rhythm.

The SCN produces a rhythm

These findings certainly indicated that the SCN is involved in the rhythmic behavior but were not conclusive evidence that the SCN itself was producing the rhythm. The evidence that the SCN is in fact the main circadian pacemaker has accumulated throughout the years. Because of the neuronal nature of the SCN, electrical signals are the first parameter to study. The first electrophysiological recordings in the SCN *in vivo* were performed using an extracellular electrode, and multiunit electrical activity was recorded. The number of electrical impulses (action potentials) per time was used as a measure of activity. The electrical activity in the SCN appeared rhythmic and is high during the day and low during the night (Inouye and Kawamura, 1979). In animals where the SCN was partially isolated from the surrounding tissue, the electrical discharge rhythm inside the SCN remained high during the projected day, while in other brain areas it was oscillating in anti-phase, with high activity during the night. In animals where the connections with the surrounding tissue were completely removed, rendering an "SCN island", the rhythm in the surrounding tissue was lost while the SCN remained rhythmic with high electrical activity during the day. This shows that the SCN drives the rhythm that was recorded outside the SCN.

SCN rhythms are autonomous

This was the first positive evidence that the SCN is in fact a pacemaker for circadian rhythms, but still not conclusive as to whether it is an autonomous oscillator. The same recording technique used in the *in vivo* study, extracellular electrophysiological recordings, could be applied *in vitro* (Figure 1.1). Small brain slices can be kept alive *in vitro* for several hours (~48h) and the tissue and

characteristics of the neurons in the slice remain relatively unchanged. It is therefore possible to record the spontaneous electrical activity in the SCN *in vitro*. Three groups independently performed the same type of recording: Brain slices containing the SCN were prepared and the electrical activity of a single neuron was recorded with an extracellular glass microelectrode. The neuronal activity was sampled from different neurons and the activity was averaged to obtain a representation of tissue-level activity (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). The averaged activity profiles all show that during the day (projected light period) the electrical activity is high, while during the night (projected dark period) the activity is low. These results could not have been the consequence of a certain day night variation in the recording since the same results were obtained from animals that had a reversed light-dark schedule. These findings indicate that the SCN does not require rhythmic input, but remains rhythmic, also when kept in constant conditions. These experiments were the first proof that the SCN oscillates endogenously.

These endogenous circadian oscillations in the SCN are present in individual cells, measurable as a circadian rhythm in electrical activity that can be measured *in vitro* (Welsh et al., 1995; Kuhlman and McMahon, 2006). When SCN cells are grown in a dispersed culture however the timing of electrical activity varies substantially between cells (Herzog et al., 1998). These results from dispersed cells show that it is important for SCN neurons to be able to communicate with each other in order to stay synchronized (Webb et al., 2009).

Circadian rhythms are generated by a molecular clock

Many cells either in the SCN or in downstream oscillators contain an intrinsic clock and have a day-night rhythm in their output. The internal cellular clock that generates these circadian oscillations has a genetic basis and functions through a molecular feedback loop. Identified genes involved in the primary negative feedback loop are *Clock* (Gekakis et al., 1998) and paralogue neuronal PAS domain-

containing protein 2, *nPas2*; *Bmal1* (Bunger et al., 2000); period homologue 1 (*Per1*) and *Per2* (Shearman et al., 1997), cryptochrome 1 (*Cry1*) and *Cry2* (Kume et al., 1999; Okamura et al., 1999; van der Horst et al., 1999). CLOCK and BMAL1 are transcription factors that together activate transcription of *Per* and *Cry* genes. PER and CRY in turn form a hetero-dimer and interact with the CLOCK-BMAL1 complex and thereby inhibit their own transcription (Shearman et al., 2000; Lee et al., 2001). The PER-CRY repressor complex is degraded over a period of time and CLOCK-BMAL1 can promote the transcription of *Per* and *Cry*. The secondary loop involves a feedback through *Rev-erbα*. Transcription of *Rev-erbα* is stimulated by the CLOCK-BMAL complex and the resulting REV-ERBα proteins complete the feedback by inhibiting transcription of the *Bmal1* gene (Preitner et al., 2002; Sato et al., 2004). The breakdown of PER and CRY is performed by casein kinase 1 delta (CSNK1D) and casein kinase 1 epsilon (CSNK1E). The speed at which PER is degraded by these kinases determines the speed of the circadian oscillation (Meng et al., 2008). These interactions between clock genes and their protein products lead to a rhythm in protein levels with a period of approximately 24h (Takahashi et al., 2008).

Interactions between Molecular clock and electrical activity

A circadian rhythm is furthermore present in membrane potential (Vm) and excitability. The circadian regulation of membrane potential and excitability in SCN neurons is controlled by the expression and modulation of different ionic channels (review see (Brown and Piggins, 2007). Membrane potential and excitability are often causally linked in neurons, but both are controlled by separate sets of ion channels. In the SCN the ionic channels known to regulate frequency of action potentials are mainly K^+-channels, like fast-delayed rectifier (fDR) K^+-channel (Itri et al., 2005), inactivating K^+-current (I_a) (Dudek et al., 1993) and Ca^{2+}-activated K^+-channels (BK and SK channels)(Pitts et al., 2006; Meredith et al., 2006; Belle et al., 2009), but Ca^{2+}-current can also affect action potential frequency (L-type

I_{Ca})(Pennartz et al., 2002). While these currents are rather well described in the SCN, the ionic mechanism of circadian membrane potential regulation has not fully been identified. Similar to molluscan circadian pacemaker neurons (Michel et al., 1999; Michel et al., 1993) a Tetraethylammonium (TEA)-sensitive K^+ current seems to form the basis of circadian Vm changes in the mammalian clock neuron (Kuhlman and McMahon, 2004). The isolation and characterization of these currents, that need to be active at resting potential, have yet to be performed, but possible candidates for this "background" current are cationic current, like Ba^{2+}-sensitive current (De Jeu and Pennartz, 2002) and currents carried by two-pore channels (Bayliss and Barrett, 2008; Linden et al., 2007). Studies on knockout models of clock genes suggest a correlation between circadian control of action potential frequency modulation and molecular machinery (Albus et al., 2002), but a real causal link has not been established and the pathways have not been identified yet.

Interestingly, there is more evidence for changes in excitability affecting clock gene expression than the other way around. Rhythmic expression of *Per* and *Tim* are suppressed in *Drosophila* pacemaker neurons when they are electrically silenced by over-expression of a leak current (Nitabach et al., 2002; Cao and Nitabach, 2008). Likewise, hyper-polarization of SCN neurons by low extracellular K^+ blocks rhythms in *Per1* and *Per2* expression (Lundkvist et al., 2005). Interestingly, not only Vm is affecting clock gene expression, also changes in excitability and synaptic input seem to have an effect on the amplitude of clock gene expression. The loss of peptidergic signaling in a knockout mouse model deficient of the receptor for vasoactive intestinal polypeptide (VIP) leads to severe loss of amplitude in the rhythmic expression of *Per2* (Maywood et al., 2006). The messenger or cytosolic pathway used to mediate membrane events to the regulation of gene expression is not yet known, but Ca^{2+} seems to be a good candidate involved in this pathway (Lundkvist and Block, 2005; Lundkvist et al., 2005).

The SCN is a pacemaker

From lesions to knock-outs

Circadian rhythms have been observed in many different physiological parameters, behavioral and cognitive functions. Moreover, many tissues and organs are able to generate a circadian rhythm (Schibler and Sassone-Corsi, 2002), provoking the question what the special role of the suprachiasmatic nucleus in this multitude of circadian oscillators may be. The prevalent view at the time is that the SCN represents a master pacemaker for the mammalian circadian system keeping most other peripheral oscillators in a strict temporal order, like a conductor giving the cue to the musicians in an orchestra (Davidson et al., 2003). To fulfill this function, the SCN rhythm itself is self-sustained and robust, well entrained to the environmental cycles and forms a uniform output signal.

In 1988, a hamster with an extremely short period of about 20h instead of the normal 24h was described (Ralph and Menaker, 1988). The mutation that this hamster carries was named tau, after the Greek letter τ, which is used to describe the period of an animal. The mutation is inherited by the offspring and a strain of these hamsters was bred for research. A spontaneously occurring mutation in the CSNK1E gene led to the shortening of the endogenous free-running period. Because of an enhanced degradation of *Per*, the duration of inhibition of its own transcription is reduced and therefore the loop is completed faster (Lowrey et al., 2000; Meng et al., 2008). The tau mutant hamsters were further studied, and used in an experiment that provides compelling evidence that the SCN is a pacemaker of circadian rhythms. SCN from these tau-mutant hamsters was transplanted into SCN lesioned wild-type hamsters and vice versa (Ralph et al., 1990). This resulted not only in a restoration of the circadian rhythm in locomotor activity, but also the free-running period and the phase of the rhythm was matching the one of the donor animal.

More evidence for the SCN as the master pacemaker comes from *in vitro* measurements of the rhythm in *Per1* gene expression in peripheral tissue compared to the SCN (Yamazaki et al., 2000). Transgenic rats were exposed to abrupt 6 h or 9 h shifts in the light-dark schedule, the tissue from SCN, liver skeletal muscle and lung was subsequently prepared and the phase of the *Per1* rhythm determined at different days after the shift. The circadian *Per1* rhythm in the SCN shifted more rapidly than the peripheral tissue rhythms supporting the idea of a hierarchically organized circadian system with the SCN as the master clock.

A number of lesion experiments have confirmed the early studies and have shown that the SCN seems to be required for a multitude of circadian rhythms in mammals. However, lesion experiments are not always conclusive, since other brain regions and pathways may be affected by the surgery complicating the interpretation of the results. While different research groups work on an SCN specific genetic deletion and have already generated a conditional knockout model for one of the clock genes (Hong et al., 2007), different models for dysfunctional SCN have been employed. The SCN can be made arrhythmic either by the use of constant light (Daan and Pittendrigh, 1976b; Witting et al., 1995; Ruby et al., 2008) or by depriving it of VIP, as an important neuro-modulator (Harmar et al., 2002; Colwell et al., 2003); review (Vosko et al., 2007). VIP is not widely distributed within the central nervous system, but predominantly found in the SCN and the cerebral cortex. This peptide seems to be critical for synchronization of neurons within the SCN and maintenance of a robust circadian rhythm in clock gene expression (Maywood et al., 2006). Mice deficient of either the peptide or its receptor show severe deficits in rhythms in behavior and physiology (Aton et al., 2005; Maywood et al., 2006).

SCN drives rhythms through electrical activity

The SCN generates different neuronal and humoral signals that could be used to transmit phase information to target tissues. Spontaneous

generation of action potentials and the circadian modulation of their frequency are hallmark features of SCN neurons. By chronically infusing tetrodotoxin (TTX), a pharmacological blocker of fast Na^+ channels, into the rat SCN, action potentials were presumably blocked, and thus electrical output was abolished (Schwartz et al., 1987). During blockage of the electrical output, behavioral activity became arrhythmic. When the blockage of electrical output was alleviated, the rhythm in behavioral activity returned in a manner that could be extrapolated from the free-running behavior before the blockage. This shows that electrical activity itself is not a prerequisite for the functioning of the clock since the rhythm during blockage was entirely free-running. Furthermore, the circadian clock in the SCN itself did not seem to be affected by the blockage of action potentials. During treatment with TTX, light entrainment capabilities were abolished. When a shift in the light dark cycle was presented during blockage of the Na^+ channels, the behavioral activity did not entrain to the new light dark regime. When the blockage was removed, normal entrainment was restored. This shows that the input to the SCN is electrical and the clock itself produces a rhythm that is synchronized to the environment through electrical communication and that electrical impulse frequency is the main output of the pacemaker (Schwartz et al., 1987).

Together it indicates that the clock mechanism functions at the molecular level, because it continues oscillating in the absence of electrical communication. On the other hand, electrical activity is necessary to reset the state and communicate the phase of the molecular clock. It was argued when the TTX experiments were performed that it is not likely that the circadian clock consisted of a single neuron with an internal clock, but instead that the neurons in the SCN communicate with each other to establish the clock output as a whole (Miller et al., 1996).

It should be noted however, that more recent studies confirmed that rhythm generation in SCN neurons can continue in the presence of TTX, but the lack of intercellular signaling does lead to de-

synchronization within the SCN and results in severe disturbances in phase accuracy in the output as a consequence (Yamaguchi et al., 2003).

Output of the SCN is electrical and humoral

The SCN has projections to many areas in the central nervous system and as a master pacemaker, it synchronizes the rhythms in peripheral tissues (Kalsbeek et al., 2006; Panda and Hogenesch, 2004). If the output pathway of the SCN is disrupted, many organs and brain areas can still express circadian rhythms as local pacemakers, although behavioral rhythms are absent. Circadian rhythms in peripheral tissues can become de-synchronized from each other and thereby overall circadian output can be abolished (Kalsbeek et al., 2008; Inouye and Kawamura, 1979; Honma et al., 1984; Eskes and Rusak, 1985; Abrahamson and Moore, 2006). These finding suggests that direct neuronal output of the SCN is important and that the SCN functions as a master circadian pacemaker.

On the other hand, in SCN lesioned, arrhythmic animals that received an encapsulated SCN graft, behavioral rhythms were restored (Silver et al., 1996). Humoral output of the SCN graft tissue can pass through the encapsulation, but electrical signaling is blocked. This shows that the humoral output drives or synchronizes the behavioral rhythms. The humoral output that is responsible for driving circadian behavioral rhythms may consist of two molecules, transforming growth factor-α (Kramer et al., 2001) and prokineticin 2 (Cheng et al., 2002; Zhou and Cheng, 2005). These two molecules have been identified as possible output from the SCN involved in regulating behavior and show that humoral output from the SCN is an important synchronizing agent for downstream structures.

Apart from these different output parameters, it is clear that the SCN control the timing of behavioral and physiological parameters through electrical and humoral output and synchronize the molecular state of peripheral oscillators (Schwartz et al., 1987; Silver et al., 1996; Kalsbeek et al., 2006). How these two different

SCN output parameters for controlling downstream oscillators interact is not clear. In the normal situation, the electrical activity within the SCN regulates the output in both electrical activity and the release of humoral factors. Therefore, the internal SCN electrical activity can be considered as a prerequisite for both output parameters.

Anatomy of the circadian visual system

The SCN in mice consists of about 10000 heterogeneous cells per nucleus (Abrahamson and Moore, 2001). By itself the clock has an endogenous rhythm close to, but not exactly 24 hours. Consequently, it needs to be adjusted to the environmental time cycle. If there are no external time cues, so-called Zeitgebers, the clock will free-run with its own period length of around 24h, and consequently shows a drift in the timing of the activity. To achieve the adjustment to the external environment, the system receives information from photic and non-photic Zeitgebers. The strongest and most obvious Zeitgeber is the light-dark cycle.

Photo receptors in the retina

The circadian system in mammals receives photic input exclusively from the eye (Nelson and Zucker, 1981; Meijer et al., 1999; Yamazaki et al., 1999). Other components are essential in communicating light information to the SCN: 1) photoreceptors in the eye, 2) retinal ganglion cells (RGC) and 3) retinal pathways.

Photoreceptors in the eye are sensitive to light of certain wavelengths and when stimulated by photic input, respond with a change in membrane potential. The change in membrane potential is transmitted to retinal ganglion cells, which in turn respond with a change in electrical discharge rate. The light information is then encoded in spiking frequency and communicated to the brain. In non-mammalian species, non-image forming photoreceptors, i.e. not involved in vision, have long been recognized (Menaker, 1971). In

many organisms, these photoreceptors play an important role in the regulation of circadian rhythms, coding for duration of light, i.e. photoperiod, and also entrainment to the environmental light dark conditions, pupillary responses and melatonin suppression. In mammals the existence of a similar, non-image forming photo pigment was predicted through experiments showing that genetically modified coneless and rodless rd/rd mice, that are visually blind and are devoid of rods and cones, were able to entrain to the external light-dark cycle and even retain a normal phase response to light (Foster et al., 1991).

Melanopsin

The expected non-image forming photo pigment was identified as melanopsin (Provencio et al., 2000). The gene coding for melanopsin was cloned and used to locate melanopsin-expressing cells in the retina. There were no melanopsin cells in the cells of the outer retina, where rods and cones are located, but instead melanopsin was present in a small number of cells in the retinal ganglion cell layer (Provencio et al., 2000; Berson et al., 2002). Although melanopsin is commonly referred to as a non-visual photo pigment, recent evidence suggests that it contributes to the functioning of the visual systems, since melanopsin containing RGCs also innervate brain nuclei where visual irradiance is encoded (Dacey et al., 2005; Barnard et al., 2006; Güler et al., 2008).

The melanopsin containing RGCs are photoreceptive at all portions of the cell and not just at a specialized photoreceptive location like in regular image forming opsins in rods and cones. The wavelength sensitivity peak appears to be at 484nm, which is comparable to the maximum sensitivity of the circadian system at the behavioral level (Takahashi et al., 1984; Provencio and Foster, 1995). The response to light is slow and the response latency decreases with increasing stimulus irradiance. When melanopsin is activated by light, it triggers a depolarization of the cell membrane through an inward current (Berson et al., 2002). The extend of the depolarization

is proportional to the stimulus energy, but the signal pathway triggering this inward current is not yet identified (Warren et al., 2003). The spike frequency seems to be related to the extend of the depolarization and maintains a plateau level during the full duration of the stimulus. After stimulus offset, the spiking activity remains for a short time while the frequency slowly decreases until the baseline membrane potential is reached (Warren et al., 2003; Morin and Allen, 2006). Besides its role as a circadian photoreceptor melanopsin also plays a role in a number of behavioral and physiological responses to light, like masking (Mrosovsky et al., 2001) and pupillary reflexes (Lucas et al., 2001). It seems that all the different photo pigments in, rods, cones and melanopsin containing cells, are required for proper functioning of these non-visual photic responses (Lucas et al., 2003; Panda et al., 2003), and that signaling from rods and cones through melanopsin containing RGCs shapes the output of the circadian photo response (Doyle et al., 2006; Güler et al., 2008).

Innervation of the SCN

The retinal ganglion cells communicate the light information via several pathways to the SCN. Most of the retinal ganglion cells directly project from the retina to the hypothalamus, and make up the RHT. The RHT consists of unmyelinated nerve fibers that project mostly to the ventral and lateral SCN cells (Morin and Allen, 2006). A small part of the RHT fibers also projects to other brain areas. Another pathway to the SCN is formed by the geniculohypothalamic tract (GHT). Some of the retinal ganglion cells that branch to the RHT also innervate the intergeniculate leaflet (IGL) and the ventral lateral geniculate nucleus (vLGN) via the retinointergeniculate tract. These parts of the geniculatum in turn project to the SCN via the GHT (Miller et al., 1996; Morin, 1994) Another part of the retinal ganglion cells project to the median and dorsal raphe nuclei. From the ventral raphe nucleus there are neural projections to the SCN but it is unclear whether these projections are directly connected to the

retinorecipient cells in the raphe nuclei (Morin, 1994; Morin and Allen, 2006).

Retinohypothalamic tract

The RHT is embedded in the optic chiasm and is a highly specialized pathway, in that it exclusively functions as a pathway mediating photic entrainment (Meijer and Rietveld, 1989). Unlike image-forming nerve fibers in the optic tract, the RHT projections to the SCN are not fully crossed in the optic chiasm but the extend is highly variable between species. The largest part of the RHT fibers end in the SCN but a small portion also innervates other brain areas (Hannibal, 2002). The RHT innervates the mouse SCN primarily in the rostral part of the ventral, ventro-medial and central portion of the nucleus, often referred to as the core. In the more caudal portion of the nucleus, the medial projections become sparse and projections primarily end in the ventro-lateral portion of the nucleus. The more dorsal part of the nucleus, or shell, receives no direct input from the RHT (Abrahamson and Moore, 2001). The communication by means of neurotransmitters between the nerve terminals originating from the RHT and the adjacent cells is also specialized; each different type of neuron has a selective range of neurotransmitters by which it communicates.

Neurotransmitters of the RHT

Neurotransmitters of the RHT are summarized by the following criteria: The neurotransmitters 1) are located in the RHT; 2) are released by light stimulation; 3) affect the cells in the SCN in a similar way as light. This functionally means that they should induce phase shifts of the endogenous rhythm, induce a change in electrical activity and stimulate light signaling pathways to induce a phase shift of the rhythm.

Glutamate and NMDA

The main neurotransmitter in the RHT is glutamate (Hannibal, 2002). Immunohistochemical studies identified glutamate immunoreactive projections from the RHT to the SCN. In the RHT the fibers that have projections to the SCN, contain glutamate, which is co-stored with pituitary adenylyl cyclase activating peptide (PACAP). Other excitatory amino acids also function as neurotransmitters like glutamate derivatives and other closely related molecules like N-methyl-D-aspartate (NMDA). L-aspartate, although not present in the RHT can also induce phase shifts in the SCN, N-acetyl-aspartylglutamate (NAAG) which is an endogenous ligand for glutamate receptors is found in the RHT but its functional significance is yet unclear.

Glutamate excites SCN cells *in vitro* and electrical stimulation of the optic nerve induces glutamate release. The circadian responses to glutamate or one of its agonists that bind to the NMDA receptor are comparable to the responses to light. This is true for both the direct electrical response and the phase response in behavior and *in vitro* (Colwell et al., 1991; Ding et al., 1994; Shibata et al., 1994). Light induced phase shifts can therefore also be blocked by application of NMDA receptor blockers (Colwell et al., 1990; de Vries et al., 1994).

PACAP

PACAP, a neuropeptide from the vasoactive intestinal polypeptide (VIP) / secretin family of peptides is co-stored with glutamate in a subset of retinal ganglion cells and in nerve terminals originating from the RHT in the ventro-lateral SCN, the retino-recipient zone (Hannibal, 2002). PACAP alters neuronal firing rate and most cells respond with a suppression in discharge frequency through the actions of PACAP on the VPAC2 receptor. A small number of cells however, respond with an excitation, probably mediated by the PAC1 receptor (Reed et al., 2002). PACAP enhances glutamatergic signaling in ventral SCN neurons. It increases glutamate release and enhances post-synaptic ionotropic glutamate receptor mediated currents

(Harrington et al., 1999; Michel et al., 2006). Functionally PACAP enhances light induced phase resetting, through the actions on glutamate release. Although PACAP by itself produces small light-like phase shifts, its primary action is most likely as a modulator of glutamate responses (Hannibal, 2006).

Substance P

Substance P, an undecapeptide is considered a neurotransmitter in both central as well as peripheral nervous system. It was thought to be a transmitter of the RHT but results are somewhat inconclusive, as it was found by tract tracing studies that Substance P containing fibers that innervated the SCN did not originate from the eye (Hannibal and Fahrenkrug, 2002). However Substance P does play a role in the SCN where it can phase shift the endogenous rhythm of the SCN *in vitro* (Shibata et al., 1992; Kim et al., 2001), however *in vivo* results are somewhat unclear as results are conflicting (Piggins and Rusak, 1997; Challet et al., 1998). Substance P is likely to play a role in mediating light induced phase shift, irrespective of whether this is intrinsic to the RHT or as an afferent transmitter in the SCN (Kim et al., 2001).

Geniculohypothalamic tract

The functional significance of the GHT in the circadian system has been studied in terms of visual properties in the SCN and behavioral consequences of disruption of the GHT. By several different techniques the input to the SCN by the GHT was disrupted, either by trans-section of the optic tracts or by bilateral lesions of the vLGN (Groos and Meijer, 1985; Inouye and Kawamura, 1982). In electrical activity recordings in the SCN of LGN lesioned animals, it was shown that SCN responses to visual stimuli were unaltered when compared to control animals with an intact GHT, therefore it can be concluded that the GHT is not necessary for tonic suppression and activation of SCN cells (Groos and Meijer, 1985). In behavior, it was shown that lesions of the LGN did not diminish normal entrainment to the light

dark cycle and show that the RHT suffices for normal entrainment. After a 12h shift of the light-dark cycle the entrainment capabilities of animals that had an LGN lesion was however significantly impaired (Rusak, 1977; Rusak and Boulos, 1981). Furthermore the magnitude of light induced phase shifts was reduced in animals with a destructed LGN (Harrington and Rusak, 1988; Harrington and Rusak, 1986; Pickard et al., 1987). In normal circumstances, the free-running period in nocturnal animals, is longer in constant light and increases with higher light intensities. In IGL ablated hamsters, these tonic effects of light are also reduced (Harrington and Rusak, 1988; Pickard et al., 1987). Taken together, these results show that the GHT is involved in controlling the phase of the SCN but do not alter the light-responses of single neurons.

In terminals of the vLGN, immunoreactivity for neuropeptide Y (NPY) was found, which suggests a possible mechanism of NPY induced phase shifts through activation of the GHT (Harrington and Rusak, 1986). It is possible that the GHT acts as a lights-off sensitive pathway (Miller et al., 1996), although the dark-pulse effects of NPY acting through the GHT are not yet fully understood.

Anatomy of the suprachiasmatic nucleus

In the SCN there are different types of cells that can be differentiated by the location in the clock, cell shape and most important transmitter content. Many receptor types that are present in SCN cells can be identified and have led to a characterization of different cell groups. Many types of glutamate receptors have been demonstrated in cells throughout the SCN but different subtypes are localized at different locations, showing that the role of different subtypes may also indicate a selective involvement in entrainment (Kopp et al., 2001).

Light response in the SCN

Retinorecipient cells in the SCN have been studied extensively by recordings of electrical activity. Retinorecipient neurons do not show a response to short light presentations but show a stable increase in

firing rate in response to a light stimulus which is maintained for the full duration of retinal illumination. Response latencies are high with an initial overshoot in spike frequency at the onset of the light stimulus (Meijer et al., 1986; Meijer et al., 1998; Drouyer et al., 2007). A small number of SCN cells are suppressed by light but show similar kinetics as light activated cells.

The response kinetics of SCN cells to light contrast those in visual brain areas involved in pattern recognition, but they are now known to correspond with the sustained increase in discharge rate in response to retinal illumination observed in melanopsin containing RGCs (Berson et al., 2002). Besides the sustained responses to light, SCN cells show a light induced increase or decrease in spike frequency as a function of the level of retinal illumination. The sensitivity of the system is dependent on the circadian time, with high sensitivity during the night and low sensitivity during the day (Meijer et al., 1986). The intensity–response curve has a sigmoid shape with no responses below a relatively high threshold. Above this lower threshold, there is an almost linear relation between increase in illumination and firing frequency. This linearity is maintained up to moderate light intensities, where the response becomes saturated (a horizontal asymptote), i.e. a further increase in luminance no longer increases the response. The relatively high intensity level of the lower threshold for a circadian light response and the saturating effects of light at moderate illumination levels suggest that SCN cells are best suited to differentiate between day and night and are most sensitive to light intensities occurring around dawn and dusk (Meijer et al., 1986; Meijer et al., 1998; Meijer, 2001).

Entrainment by light

The endogenous period τ of the circadian rhythm, which deviates slightly from 24h, is synchronized to the environment through the phase dependent shifting effects of light. The endogenous state of the pacemaker, in circadian time (CT) or phase φ of the pacemaker, can only be seen in the absence of external Zeitgebers. In the presence of

external synchronizers, the phase of the clock can be referred to as Zeitgeber time (ZT). In nocturnal animals, activity onset is defined as CT 12 when the animal is in constant conditions. ZT 12 is defined as lights off, which roughly corresponds to activity onset but may not reflect the true phase φ of the pacemaker. The phase shifting actions of light enable the organism to synchronize the internal timing to the environment. Phase shifts in behavior take several daily cycles to complete and show a transient transition to its new phase. The state of the pacemaker however is directly shifted after a light pulse, as was demonstrated by a double pulse paradigm (Best et al., 1999; Watanabe et al., 2001). Two discrete light pulses were administered and the resulting phase shift after the two pulses was used to calculate the shift induced by the first pulse. Depending on the direction, speed and magnitude of the phase shift induced by the first pulse, the second pulse will hit another area on the phase response curve when compared to a situation without the first pulse. More recently it was found that the phase of the SCN as a whole may not be fully shifted after application of a light pulse, as was found after a shift in the light-dark cycle. The circadian rhythm in the SCN showed regional differences (Vansteensel et al., 2003b; Albus et al., 2005) and even different phase shifting responses between clock genes (Reddy et al., 2002).

The phase shifting effects of light furthermore enable an organism to adapt to seasonal changes in the duration of light, or photoperiod. The duration of daylight in the different seasons has marked effects on the behavior and physiology in many mammals. The duration of light alone is sufficient for animals to show seasonal responses. These seasonal responses are mediated by the SCN and are independent of temperature changes. For instance hamsters that are kept on photoperiods with less than 12h light per day will develop a winter coat irrespective of ambient temperature and may go into some sort of hibernation, which is a physiological response to minimize energy expenditure (Goldman, 2001). Several photoperiodic responses are mediated by melatonin, primarily produced by the pineal gland.

These photoperiodic changes in melatonin excretion are driven by the SCN (Johnston, 2005). The photoperiodic changes in the SCN are visible as an increase or decrease of the duration of elevated electrical activity (Mrugala et al., 2000).

The SCN as a Neuronal Network

The circadian rhythm in the SCN has a single peak each circadian cycle. There are basically two approaches that can enable the clock to produce its monophasic output. One view is that all neurons in the SCN have synchronized phasing and produce a coherent rhythm in their output, small cellular differences in phasing would be averaged in the total clock output (Liu et al., 1997b; Low-Zeddies and Takahashi, 2001; Herzog et al., 1998). The other view is that the clock produces a rhythm based on the combined output from multiphasic rhythmic cells. Electrical discharge rhythms in the SCN entrain to the external light-dark cycle and reflect the photoperiod in duration of elevated electrical discharge. These rhythms are not constrained to a monophasic time-point but may also oscillate in anti-phase and the phase relationships between cells can be remodeled by the environment to enable entrainment (Pittendrigh and Daan, 1976b; Jagota et al., 2000; Mrugala et al., 2000). In 2003 the latter view of clock organization was confirmed by three research groups independently and has had great implications for future research on communication and organization of the biological clock.

Animals that were genetically altered to express a short half-life green fluorescent protein (GFP), that was coupled to the *Per1*-promotor, were recorded *in vitro* to investigate the organization of individual oscillators in the clock (Quintero et al., 2003). Quantitative imaging revealed that the per1 promoter had its peak activity 2 hours before lights off, regardless of the time of preparation. Therefore, these rhythms were not induced by the preparation procedure. The timing of the second peak after the first peak in fluorescence *in vitro* was about 23.5h. In the absence of Zeitgebers *in vitro*, the period

length was comparable to the period of behavioral rhythms in mice in constant darkness. Furthermore, electrical activity of *Per1* expressing cells was recorded and there was a strong positive correlation between spike frequency and *Per1* expression. In addition to the whole SCN recordings of fluorescence, individual neurons were imaged and it was observed that about 90% of the neurons showed rhythmic activity. A minority of 11% of the cells did not show any rhythmic *Per1* driven GFP when the slice was taken from a mouse kept in an LD cycle. When cells were taken from animals kept in DD, 26% of the cells did not show a rhythm in *Per1* expression. This shows that some of the cells recorded directly after an LD cycle may have been driven by the light-dark cycle. The percentage of rhythmic cells was different for different regions within the SCN. Further investigation of the timing of peak fluorescence revealed that about 50% of the cells had their peak in synchrony with the output of the whole SCN peak, both in LD as well as in DD. The timing of the peaks in slices from LD and DD differed somewhat and Per expression was delayed in slices from DD conditions, which may be due to the direct effects of light on *Per* (Asai et al., 2001). The single unit peaks that were outside the main peak were clustered in time and showed regional differences, with a medial to lateral gradient in peak times, early cells were mostly located in the medial SCN and peaked around ZT 5, whereas the latest peaks in Per1 fluorescence was in lateral cells around ZT18.

Another group recorded from organotypic brain slice cultures from mice carrying a firefly luciferase gene that was coupled to the *mPer1*-promoter (*mPer1-luc*), to investigate the network interactions of the autonomous molecular clocks in SCN cells and how they underlie the tissue oscillator (Yamaguchi et al., 2003). They were able to track the peak in *mPer1*-promoter activation of hundreds of cells simultaneously and found that about 99% of the cells showed a rhythm in bioluminescence. Furthermore, they found that peak and trough times in *Per* luciferase expression differed substantially between cells, even when they were located close to each other. These phase differences between cells remained constant over the cycles and

showed an anatomical preference, dorso-medial cells were first, then central cells and finally ventral SCN cells. To see if the dorso-medial cells were driving the ventral rhythm, a knife-cut was made to separate dorsal from ventral cells. In the dorsal part, the cells remained rhythmic, but lost synchrony. In the ventral part the cells remained rhythmic and synchronized. The data show that the dorsal cells are not driving the rhythm in the ventral part. The rhythmic properties of the clock are caused by an interlocked feed-back loop of protein levels that feed back on the transcription for other genes, thereby creating an oscillation in protein levels. When all protein synthesis, including luciferase, was blocked by application of cycloheximide (CHX) the level of bioluminescence decreased as a result. After full PER depletion, (36h of CHX), the entire clock was stopped and there were no detectable levels of clock proteins left. When the application of CHX stopped, and protein synthesis continued, all cells simultaneously started the molecular feedback loop, resulting in a completely synchronized rhythm. The first peaks in bioluminescence appeared 3h after restoration of protein synthesis, phase differences in peak expression between cells gradually restored over a time span of 4-5 days.

Because intracellular communication takes place via electrical activity, Na^+ channels were blocked by application of TTX, resulting in a loss of action potentials. After a few days, the individual *Per* oscillations continued at a lower amplitude and became desynchronized relative to one another, resulting in an overall lack of a rhythm. When TTX was removed, the preceding phase relations between individual clock cells were restored. These data together suggest that inter-neuronal signaling keeps stable phase relations between cells, and is needed for high amplitude molecular rhythms.

In another study performed in 2003 (see chapter 3) recordings of extracellular electrical activity were performed in rat brain slices. In these multiunit recordings, the peak in electrical activity was at midday. By an offline amplitude selection of action potentials, the area around the electrode could be selected and enabled the selection

of small populations of neurons. The subpopulation activity revealed short durations of increased electrical activity, for small populations and when a larger population was selected, the width of the signal increased and the peak phase stabilized towards midday. Furthermore, electrical activity of single units was extracted from the multiunit signal, and revealed that individual neurons display only short durations of activity. It was proposed that these short durations of activity and heterogeneous phasing might contribute to photoperiod encoding. Simulations were performed with an average single unit activity pattern, distributed linearly over the photoperiod. These simulations showed that the phase distribution of single units could explain the increase in peak width in an increased duration of light.

Together, these three studies showed that the SCN is composed out of differently phased autonomous clocks, and that the phase differences are an intrinsic property of the network of cells. The differences in timing, number of autonomously rhythmic cells and their anatomical location may have been due to differences in techniques. But what is consistent between these studies is that the phasing of individual neurons is the composite of many different phases and that these different phases are an intrinsic property with possibly differential coupling mechanism, that may be region specific. The observed phase differences may be an essential autonomous anatomical property, partly driven by light activation.

Introduction to research questions

In the present thesis, the functional significance of phase differences between SCN neurons is investigated. It is proposed that these phase differences are essential for photoperiodic encoding in the SCN. In chapter 2, we evaluate the acute brain slice recording methodology for circadian rhythm research. The acute brain slice preparation is extremely useful in the study of circadian properties as the aftereffects of environmental conditions can be evaluated *ex vivo*. The studies described in chapter 2, are aimed to elucidate whether the preparation of the brain slice introduces changes in the peak time and

waveform of the electrical activity rhythm. The results show that the peak time and waveform are robust and that he time of slice preparation, relative to the circadian cycle, does not affect the basic properties of the rhythm. The results are important for the chapters following chapter 2, in which the organization of the SCN in long and short photoperiods is evaluated ex-vivo.

In chapter 3, we investigated how the electrical activity of a single neuron contributes to the output signal of the large population of neurons in the SCN. We prepared hypothalamic brain slices from rats, containing the SCN with the technique covered in chapter 2. From the recorded multiunit data, we extracted the activity of subpopulations and single units. We found evidence of single units and subpopulations that are active for only a relatively short time and activity peaks are distributed over the 24h period. The output of the short active and differently timed subpopulations and single units together form a seemingly coherent, monophasic waveform at multiunit level. The observed short duration of electrical activity of single units and subpopulations is further explored and a simulation is presented on the basis of the recorded data. These simulations form the practical illustration of the hypothesis that the distribution over time of the observed single units lies on the basis of photoperiodic encoding in the SCN.

In chapter 4 we further investigated the hypothesis that the short duration of activity of single units and subpopulations play a role in encoding day length in the SCN. We recorded wheel-running activity of mice in long and short photoperiods and found that in behavior the day length is encoded. We furthermore recorded *in vivo* multiunit electrical activity and found that the *in vivo* multiunit electrical activity also reflects the photoperiod the animals are entrained to. Finally, *in vitro* recordings of multiunit electrical activity were performed to investigate the role of single units and subpopulations in photoperiodic encoding by the SCN.

In literature there has been a notion that the phase shifting capacity in long day length was significantly reduced and in short day

length it was enhanced. Systematic analysis of this phenomenon was lacking. In chapter 5, we present a functional mechanism underlying these observations. We created a phase response curve to light for mice entrained to short and long photoperiods. We found that in these mice the phase shifting capacity in short days was indeed enhanced whereas in long day length it was reduced. We then investigated whether these changes in responsiveness may have been caused by a desensitization of retinal input to the SCN or are an intrinsic property of the SCN. We adjusted the light exposure during entrainment so that the animals from both day lengths received an equal amount of photons each day, and found that this did not change the responsiveness to a light pulse in any circumstance. To study this effect in more detail, we recorded electrical activity in brain slices from animals entrained to both photoperiods and applied NMDA to mimic a light pulse. We found that the different responses are intrinsic to the SCN and we present a functional mechanism that underlies these changes.

In chapter 6, we discuss the preceding chapters in the context of more recent findings. We look into possible research perspectives and link our findings to other research. A major conclusion is that the neuronal network of the suprachiasmatic nucleus is of key importance to its function, and that the network properties of the SCN shape the output.