INTRODUCTION

The rotation of earth around its axis and the sun generates rhythmic daily and seasonal cycles in our world. These rhythms affect the quality of life in different manners. Animals have evolved to be active in the night (nocturnal) or the day (diurnal) according to their anatomical and physiological features, source and availability of food and presence of predators or prey. They have also developed various seasonal strategies such as hibernation and migration to survive the winter when there is not enough food sources available. Most animals are able to time the mating behavior to increase the chance of survival for their offspring. Thus, from an evolutionary point of view it is crucial for any organism to predict and follow environmental cyclic rhythms. However, for many years the general opinion was that all rhythms in animal behavior are caused by environmental cues, such as day light, various length of the day in different seasons, temperature and magnetic field of the earth. In 1729 de Mairan noticed for the first time a daily rhythm in *Mimosa pudica*, a plant which opens its leaves during the day and closes it at night. He hypothesizes that day light triggers this behavior in the *Mimosa*. To test his hypothesis, he exposed the plant to the constant darkness (DD) and recorded the behavior. Notably, the daily rhythmic opening and closing of the leaves persisted even in the absence of sunlight. However, his main conclusion was that other factors than sunlight, such as temperature and magnetic fields, were responsible for the rhythmic behavior. De Mairan's work stimulated further research in the field of chronobiology. However, it took more than two centuries before the concept of an endogenous time keeper was accepted.

In 1832 de Candolle showed that the movement of the leaves continues in constant conditions. In addition, he found that the leaves open an hour earlier each day, thus indicating a period of 23 hours (h) in the absence of the sunlight. This phenomenon is called "free running" and indicates the period of the endogenous clock slightly deviates from 24h. Therefore the environmental light/dark cycle is required to entrain the internal clock every day. Many years later in 1959 the word **circadian** was introduced to describe the approximately 24h cycles which are generated endogenously by every organism. Circadian is a Latin word composed of circa = around and dies = day which means "approximately a day" (Halberg, 1959).

In 1910, by an accidental observation Forel suggested that animals have a memory of time and can measure the passage of the time. In his summer house, Forel usually had breakfast outside on the terrace around the same time of the day and there appeared some bees to collect marmalade. After couple of days it was impossible to have breakfast outside as many bees appear on the terrace slightly before breakfast time. This bee appearance on the terrace around breakfast time continued for couple of days without any marmalade or other food being presented. However, bees were not present on the terrace at other time points of the day. In 1929, von Frisch and Beling showed in a systematic experiment that the organisms can truly measure the passage of time (Beling, 1929). This observation directed scientists to the hypothesis that circadian rhythms are outputs of an internal system measuring time.

In 1935 Bünning demonstrated that plants and insects raised in constant conditions still display a circadian rhythm and concluded that circadian rhythms are genetically inherited. In 1950s, Colin Pittendrigh performed many studies to convince biologists that a circadian system generates the timekeeping features of the organisms. Since then, many researchers documented that circadian rhythms exist in many organisms from unicellular to higher species including human.

First evidence for the location of mammalian biological clock was found in 1972 by two independent research groups. By making small brain lesions in anterior hypothalamus directly above the optic chiasm, they demonstrated disappearance of rhythmicity in wheel running and drinking behaviors and hormone secretion (Stephan and Zucker; Moore and Eichler, 1972). In 1979 Inouye and Kawamura for the first time implanted an electrode in the rat suprachiasmatic nucleus (SCN) and recorded the rhythmic SCN electrical activity with a peak around the midday while other brain regions oscillating in anti-phase i.e. with a peak of electrical activity at night. When the SCN was partially dissociated from the surrounding tissues by anatomical dissections, the electrical rhythm remained stable both in the SCN and surrounding tissues. However, only after complete anatomical isolation of the SCN, surrounding tissues lost their rhythm and only the SCN remained rhythmic. It lasted until 1982, when it was finally accepted that SCN is able to generate an electrical activity rhythm independently from synaptic inputs from other brain regions. Three different research groups succeeded to isolate the SCN as a brain slice and recorded its rhythmic electrical activity *in vitro* (Green and Gillette, 1982; Groos and Hendriks, 1982; Shibata et al., 1982). In 1987 Schwartz and colleagues showed that blocking the SCN electrical activity by tetrodotoxin (TTX), a pharmacological blocker of fast sodium channels, inhibits behavioral rhythmicity and re-entrainment of the SCN to a shifted light/dark cycle. This data indicates that SCN input and output signals are mediated by its electrical activity. Further evidence that SCN is the main circadian pacemaker has been compiled in the last decades. Since 1979, many research groups confirmed that disruption of SCN output results in behavioral arrhythmicity, although many organs and some brain areas remain rhythmic as local pacemakers. However, peripheral circadian rhythms desynchronize from each other and the overall circadian output is abolished in the absence of SCN output (Inouye and Kawamura, 1979; Shibata et al., 1982; Honma et al., 1984; Eskes and Rusak, 1985; Panda and Hogenesch, 2004; Abrahamson and Moore, 2006) and pinpoints the SCN as a master circadian pacemaker. The role of the SCN is to generate a precise image of the solar time and then convey that across the brain and body via hormonal and neural pathways to time the physiological and behavioral aspects of the organisms.

SUPRACHIASMATIC NUCLEUS AS MAMMALIAN CIRCADIAN CLOCK

The SCN is situated in the anterior hypothalamus above the optic chiasm and bilateral to the third ventricle and interacts with many brain regions (**Fig. 1A**). It receives light input directly from melanopsin-containing retinal ganglion cells via retinohypothalamic tract (RHT; Hattar et al., 2002; Morin and Allen, 2006) and in turn synchronizes other brain regions and downstream peripheral targets to the light/dark cycle (Gachon et al., 2004). Retinal projections release glutamate and pituitary adenylate cyclase-activating polypeptide (PACAP, as a neuromodulator) at synaptic terminals to the SCN (Morin and Allen, 2006). Anatomically, the SCN is composed of ventrolateral region (core) which is retionrecipient and dorsomedial region (shell) which receives input from the core, while the core is only sparsely innervated by the shell (Albus et al., 2005). Both ventrolateral and dorsomedial SCN regions consist of different cell types. Vasoactive intestinal peptide- (VIP) and gastrin-releasing peptide- (GRP) producing cells are found in the ventral area. Argenine vasopressin- (AVP) and somatostatin-containing cells are mainly located dorsally (Abrahamson and Moore, 2006; **Fig. 1B**). VIP and AVP are colocalized with GABA in 38% and 15% of synaptic terminals respectively (Buijs et al., 1995). Most, if not all of the SCN neurons express GABA receptors and generate spontaneous post synaptic GABAergic currents (Moore and Speh, 1993). To a certain extent, SCN anatomy and neuropeptides is specie-specific and might be more complex than simply being divided in to core/shell regions (Morin et al., 2006).

SCN NEURONS AS AUTONOMOUS CIRCADIAN OSCILLATORS

SCN cells generate rhythmic oscillations in gene expression and neuronal activity. They are autonomic neurons, which stay rhythmic even when they are isolated from the network of cells (Herzog et al., 1998; Liu et al., 2007; Webb et al., 2009). The electrical impulse of the SCN peaks in the middle of the day and is the main circadian output to downstream targets (Colwell, 2011). However, it is not the actual oscillatory mechanism of the SCN. The rhythm in neuronal electrical activity is controlled by other rhythmic components such as different ionic currents (Brown and Piggins, 2007). At the molecular level, the core circadian clock is driven by numerous clock genes that are rhythmically expressed with a period of about 24h.

In 1971 the first clock gene was discovered in *Drosophila* which was called "Period" (Konopka and Benzer, 1971). After the discovery of the first clock gene, the gene network underlying the molecular clock has been largely elucidated. The molecular clock enables cell autonomy within the SCN based on a negative transcriptional-translational feedback loop. The loop starts when CLOCK/BMAL1 dimers bind to the E-box regions in the promoter of clock genes thereby inducing the transcription

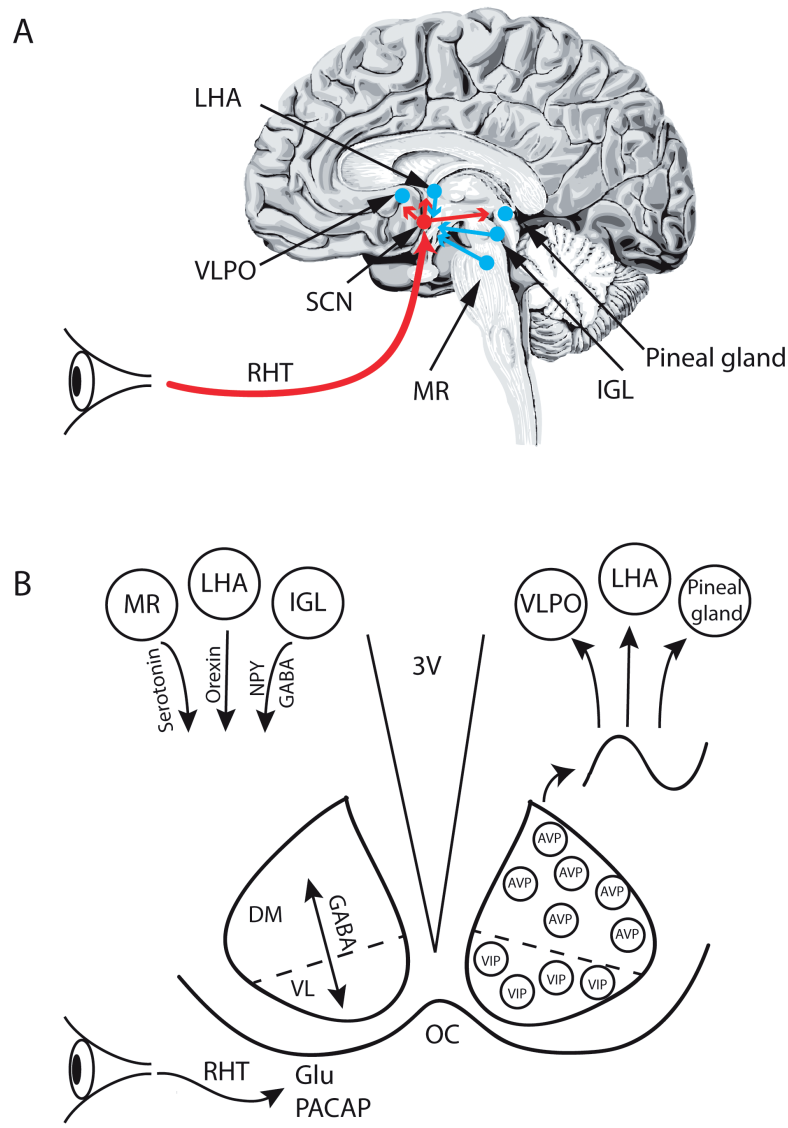

Figure 1. The location and organization of the suprachiasmatic nucleus (SCN). (**A**) The SCN is located in the anterior hypothalamus. Light information is transmitted to the SCN via the retinohypothalamic tract (RHT) which releases glutamate (Glu) and pituitary adenylate cyclase–activating polypeptide (PACAP). Several brain nuclei receive SCN output (red arrows) and/or project to the SCN (blue arrows), some involved in sleep/waking regulation (VLPO, LHA) and melatonin release (pineal gland). (**B**) The SCN is functionally organized in at least two distinct regions: dorsomedial (DM) and ventrolateral (VL) areas. Argenine vasopressin (AVP) producing cells mark the dorsomedial area and vasoactive intestinal peptide (VIP) producing cells are mainly found in the ventrolateral area. These neuropeptides, in concert with GABA, are involved in synchronization and neuronal interaction within the SCN. The SCN receives different inputs (left) and transmit its output to several brain nuclei and downstream targets (right). 3V = third ventricle, IGL = intergeniculate leaflet, LHA = lateral hypothalamus, MR = median raphe, neuropeptide Y = NPY, OC = optic chiasm, VLPO = ventrolateral preoptic nucleus.

of period (*Per1*, *Per2* and *Per3*) and cryptochrome (*Cry1* and *Cry2*) genes (Koike et al., 2012; Huang et al., 2012). In turn, PER and CRY proteins translocate to the nucleus and inhibit the transcription of genes. Degradation of PER and CRY permits a new cycle. Casein kinase 1 delta plays an important role in maintaining the 24-h circadian period by phosphorylating the core clock proteins such as PER (Vielhaber et al., 2000; Meng et al., 2008; Etchegaray et al., 2009; Lee et al., 2009). The amount of BMAL and CLOCK is also rhythmically controlled by an additional feedback loop including *Rev-Erb*α, *Rev-Erb*β and *ROR* genes, which are driven by *Clock-Bmal1*, and their protein products promote rhythmic expression of *Bmal1* (Preitner et al., 2002). Many genes that rhythmically control excitability and secretion, are regulated by these negative feedback loops. To maintain the cell autonomy there should be a link between membrane and nuclear processes. It is not completely known that how this molecular circadian clock is connected to rhythmic membrane properties.

CIRCADIAN CONTROLLED IONIC CONDUCTANCES

Rhythmic electrical activity is an important output of the SCN that controls behavioral and physiological phenomena. Both in nocturnal and diurnal species, the electrical activity of SCN neurons peaks in the middle of the day when most of the SCN neurons actively generate action potentials (AP) with a high rate. In the night the same cells are either silent or fire action potentials with lower frequencies (**Fig. 2**). This rhythm in electrical activity is regulated by two main classes of ionic conductances which are mostly circadian controlled as well (Brown and Piggins, 2007). Firstly, currents responsible for the excitatory drive such as persistent sodium (Na^+) and hyperpolarization-activated currents. Secondly, currents which translate the excitatory drive to action potentials and control firing frequencies e.g. different classes of potassium (K^+) currents (Colwell, 2011).

Persistent Na^+ currents

During the day, SCN neurons have a significantly more depolarized resting membrane potential which is very close to the threshold for initiating an action potential (Kuhlman and McMahon, 2004). One of the excitatory drives, bringing SCN neurons from resting to threshold values, are Na^+ persistent currents, which are activated between -60 to -40 mV (Jackson et al., 2004). No circadian regulation of this current has been reported so far. However, closure of these channels prevents the daily rhythm of the SCN electrical activity (Kononenko et al., 2004).

Hyperpolarization-activated currents

In response to hyperpolarization, almost all SCN neurons generate a depolarizing current. When the hyperpolarization-activated channels are open, Na^+ enters and K^+ leaves the cell but the net result is membrane depolarization (de Jeu and Pennartz, 1997). Recently,

it was shown that the magnitude of this current shows a modest circadian rhythm with a peak during the day-time when the electrical activity is high (Atkinson et al., 2011). Hence, these channels may contribute to the excitatory drive in the SCN cell membrane and prepare it for generating action potentials mainly during the day.

K^+ Currents

Among the major classes of K^+ channels, two are expressed widely in the SCN: voltage-gated and calcium (Ca^{2+}) activated K^+ channels. Both are involved in repolarization and afterhyperpolarization (AHP) of an action potential. It has been shown that several of the currents belonging to these families represent a circadian rhythm in their current magnitude (**Fig. 2**). Fast delayed rectifier (FDR) and A-type (I_A) K^+ currents for example are larger during the day when the electrical activity increases (Itri et al., 2005; Itri et al., 2010). FDR current has been shown in different brain regions that is crucial for creating high frequency sustained train of action potentials (Rudy and McBain, 2001). I_A seems to serve a similar function during the day in the SCN: in concert with FDR these currents facilitate the generation of action potentials and increase the electrical activity. These currents shape the action potential waveform with a rapid repolarization, which prepares the membrane to trigger the next action potential. In the night when the electrical activity declines, I_A and FDR currents both decrease in magnitude.

In contrast to FDR and I_A currents, large conductance Ca^{2+} activated K^+ currents (BK) increase in magnitude as well as protein expression at night (Pitts et al., 2006;

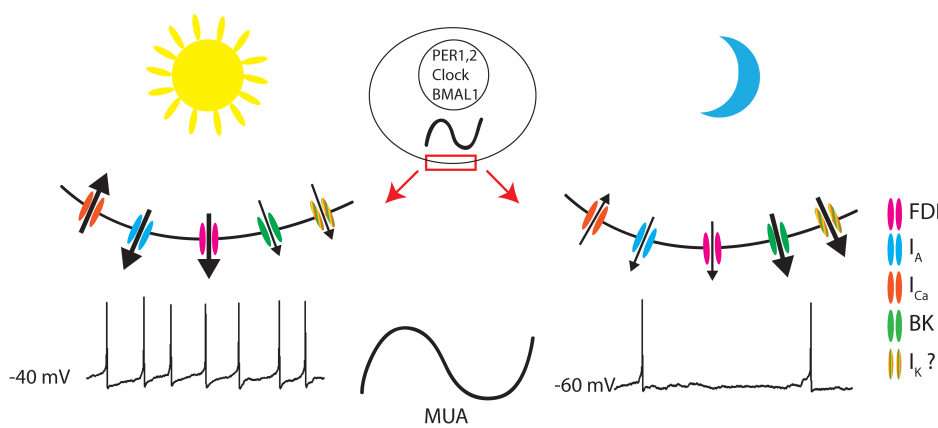

Figure 2. Circadian controlled ionic currents in the suprachiasmatic nucleus (SCN). The clock-controlled fast delayed rectifier (FDR), A-type K^+ currents (I_A), large conductance Ca^{2+} activated K^+ currents (BK) and voltage-dependent Ca^{2+} currents (I_{Ca}) modulate membrane excitability in a circadian manner. This results in a rhythm in the multi-unit activity (MUA) of the SCN with higher frequency of action potential during the day compared to the night. A yet to be identified K^+ current (I_K) contributes to regulation of membrane potential, which is more depolarized during the day as compared with the night. The thickness of the arrows illustrates the magnitude of the current that passes through channels.

Kent and Meredith, 2008). BK currents are involved in the AHP phase of APs within the SCN and other brain regions. Activation of these channels decreases the probability of next action potential generation in the cell and reduces cell excitability (Montgomery and Meredith, 2012). Therefore BK channels are important for night-time suppression of SCN electrical activity. Recently, a bidirectional role for BK channels in the SCN was suggested. Fast activation of these channels causes a rapid repolarization, recovers the Na^+ channels from inactivation and leads to membrane excitation (Montgomery and Meredith, 2012). This paradoxical impact of BK channels on electrical activity and AP waveform in the SCN is not completely understood and the role of BK channels in the SCN may differ in various phases or physiological and pathological conditions.

Apart from electrical activity, resting membrane potential (RMP) is also rhythmically controlled (**Fig. 2**). A tetraethylammonium (TEA)-sensitive K^+ current is responsible for night-time membrane hyperpolarization (De Jeu et al., 2002; Kuhlman and McMahon, 2004). This K^+ current is larger at night and causes a higher membrane conductance and consequently lower input resistance in the SCN cells in this phase of cycle (Kuhlman and McMahon, 2004). In contrast, during the day closure of these K^+ channels reduces the membrane conductance and increases the input resistance. Two-pore-domain potassium (K2P) channels are possible candidates for RMP regulator as some of K2P genes such as, TWIK1, TASK1, TREK1, and TASK3 are expressed rhythmically in the SCN and in *Drosophila* circadian pacemaker neurons (Talley et al., 2001; Panda et al., 2002; Lein et al., 2007). K2P channels carry a leak K^+ current and have little voltage dependency. They are expressed throughout the central nervous system and contribute to the resting membrane potential of many types of neurons (Millar et al., 2000; Talley et al., 2000). Notably, K2P expression in the ventral lateral neurons (LNvs) of *Drosophila* hyperpolarized the resting membrane potential of cells in the day (Sheeba et al., 2008). The depolarized resting membrane potential of SCN neurons during the day could be due to changes in kinetic, density or expression of K2P channels, or other yet to be described channels involved in regulating the RMP in the SCN.

Ca^{2+} currents

Various kinds of voltage-gated Ca^{2+} channels including L-, P/Q-, T-, R- and N-type channels are expressed in the SCN. L-type channels are highly expressed in the SCN where R- and N-type channels show a low expression and P/Q- and T-type channels are moderately expressed (Nahm et al., 2005). P/Q-type Ca^{2+} channels are located presynaptically in the SCN (Nahm et al., 2005). They play a crucial role in neurotransmitter release (Wu et al., 1999) and in mediating the signals of central nervous system to the dorsal SCN (van Oosterhout et al., 2008). Voltage-gated Ca^{2+} channels mediate light-induced phase shifts in the SCN (Kim et al., 2005) and maintain rhythmic expression of clock-genes (Lundkvist et al., 2005).

The magnitude of the L-type Ca^{2+} current is higher during the day when a high frequency of electrical activity is generated in the SCN (Pennartz et al., 2002). Moreover,

the baseline intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ of the SCN cells is elevated during the day compare to the night (Colwell, 2000). Calcium influx via the L-type channels could partially be responsible for the day-time elevation of $[Ca^{2+}]_i$. Moreover, Ryanodine and inositol (1,4,5)-triphosphate receptors, located in the membrane of intracellular structures, are also involved in $[Ca^{2+}]_i$ homeostasis within the SCN neurons. Ryanodine receptors exhibit a circadian rhythm in protein expression with a peak in subjective day when the $[Ca^{2+}]_i$ is high (Diaz-Munoz et al., 1999) .

It is known that Ca^{2+} and BK channels are colocalized in cell membrane. BK channels apart from being voltage dependent, also require a rise in intracellular Ca^{2+} for their activation. However in the SCN the BK current peaks at night when the $[Ca^{2+}]_i$ decreases. The reason for this reversed correlation between BK magnitude and Ca^{2+} concentration in the SCN needs to be further investigated.

SCN NEURONS AS A NEURAL MULTI-OSCILLATOR NETWORK

Single SCN neurons exhibit a circadian rhythmicity and do not need a rhythmic input to generate circadian rhythms (Webb et al., 2009). However, single SCN neurons are not sufficient to establish all the features of the circadian clock. The SCN therefore, is a multi-oscillator structure in which interneuronal phase synchrony leads to a coherent rhythm. Synchronization among the SCN neurons is determined by a variety of coupling mechanisms including gap junctions, neurotransmitters and neuropeptides, which enable the system to deal with the environmental challenges in a flexible manner. Here, the role of two main signaling molecules i.e. VIP and GABA in coupling mechanisms will be discussed in more details.

VIP

There is some evidence that expression levels of VIP mRNA and peptide exhibit a diurnal oscillation but do not show a circadian rhythm in constant darkness (Shinohara et al., 1993; Ban et al., 1997; Shinohara et al., 1999). However, VIP release from rat SCN slice cultures has a circadian oscillation that continues for a number of cycles in constant conditions (Shinohara et al., 2000). Moreover, VIP mRNA shows a circadian rhythm in the mouse SCN (Dardente et al., 2004). These rhythms may be important for outputs from the circadian system since VIP acts as one of the major synchronizing factors within the SCN network. When VIP or its receptor VPAC2 are genetically deleted, circadian rhythmicity in behavior, electrical activity, gene expression and metabolism will be weakened and SCN loses its synchrony to environmental light cues (Harmar et al., 2002; Colwell et al., 2003; Aton et al., 2005; Maywood et al., 2006; Bechtold et al., 2008). Therefore, VIP is crucial for intercellular communication and synchronization between SCN neurons. Loss of VPAC2 receptor however, demonstrates more dramatic desynchrony within the SCN network compare to lack of VIP (Colwell et al., 2003;

Brown et al., 2007). This indicates a compensatory role for other neurotransmitters such as PACAP that binds with the same affinity of VIP to VPAC2 receptors (Gottschall et al., 1990; Morrow et al., 1993). Furthermore GRP synchronizes the SCN network when VIP signaling does not function properly (Brown et al., 2005; Maywood et al., 2006).

GABA

The majority of SCN neurons (more than 90%) contain GABA in their synaptic terminals and express GABA receptors in their membrane (Moore and Speh, 1993; Moore et al., 2002). In the SCN, the amount of GABAergic spontaneous activity shows a circadian rhythm with a peak at early night (Itri et al., 2004). When the $GABA_A$ receptor is activated an intrinsic GABA-gated chloride (Cl^-) channel will be opened. The subsequent Cl^- influx results in an inhibitory action of GABA by membrane hyperpolarization (Kaila, 1994). It is important to note that the intracellular chloride concentration $[Cl^-]_i$ determines the direction of Cl^- movement through the channel. In normal adult neurons $[Cl^-]_i$ is low. Therefore, opening of the Cl^- channel leads to Cl^- influx and hyperpolarization (**Fig. 3A**). Different classes of Cl^- pumps are involved in regulation of $[Cl^-]_i$. Two different type of these pumps are expressed in the SCN: The Na^+K^+-$2Cl^-$ cotransporter (NKCC) and K^+-Cl^- cotransporter (KCC; Belenky et al., 2008). NKCC1 transports Cl^- into the cell and increases the intracellular Cl^- concentration. In contrast KCC2 keeps the intracellular Cl^- concentration low. The balance between the activity of the two transporters defines the equilibrium potential of Cl^- (E_{Cl}) in the SCN cells. Recently it has been shown that the mechanisms by which Cl^- gradient is established in neurons are more complicated to be determined only by theses cotransporters and local impermeant anions may also be important for $[Cl^-]_i$ (Glykys et al., 2014). Nevertheless, if E_{Cl} is depolarized, because of a higher Cl^- concentration inside the cell, Cl^- flows out of the cell and the membrane potential will be depolarized (Cherubini et al., 1991).

In 1997, it was reported that in the SCN neurons of healthy adult rats, GABA apart from its traditional inhibitory function acts also as an excitatory neurotransmitter (Wagner et al., 1997). Since then, many other labs confirmed the dual effect of GABA on SCN neurons, which depends on the time of the day, regional localization and the activity state (De Jeu and Pennartz, 2002; Albus et al., 2005; Choi et al., 2008; Irwin and Allen, 2009). A higher activity of NKCC1 generating a depolarized E_{Cl} may underlie the observed GABA-mediated excitation (Choi et al., 2008; Belenky et al., 2010; **Fig. 3B**). It has been shown that expression of NKCC1 but not KCC2 may be under circadian control in the SCN (Panda et al., 2002). The functional role of the GABA-mediated excitation in the adult SCN is not known yet. It is suggested that GABA-mediated excitation may relay the photic and phase information from ventral to dorsal SCN following a shifted light/dark cycle (Albus et al., 2005). GABA is essential for intercellular and interregional communication within the SCN network (Liu and Reppert, 2000; Albus et al., 2005; Han et al., 2012). However, in VIP knock-out animals, blockade of endogenous GABA does not impair SCN synchrony but improves it (Aton et al., 2006; Freeman, Jr. et al., 2013).

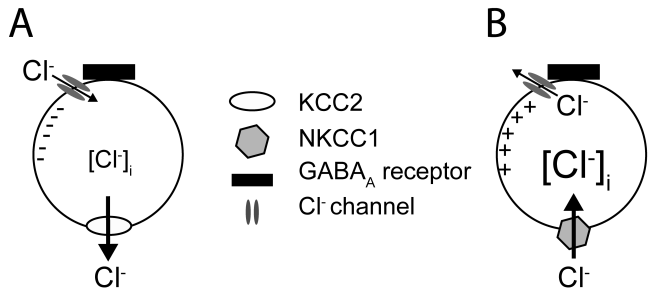

Figure 3. Dual function of GABA as an inhibitory and/or excitatory neurotransmitter. (**A**) in normal adult brain KCC2 cotransporter drives the Cl^- out of the cell and maintains the intracellular Cl^- concentration ($[Cl^-]_i$) in low levels. When GABA receptor is activated the GABA-gated Cl^- channel will be opened and Cl^- influx results in membrane hyperpolarization. (**B**) in immature neurons as well as in some adult neurons in specific brain regions e.g. SCN GABA acts as an excitatory neurotransmitter due to the function of NKCC1 cotransporter. NKCC1 transports the Cl^- into the cell and increases the $[Cl^-]_i$. Consequently when the GABA -gated Cl^- channel is opened, Cl^- flows out and membrane will be depolarized.

This destabilizing effect of GABA was considered as a necessary component for resetting the network to a new phase (Freeman, Jr. et al., 2013). It is shown that VIP affects GABA signaling in different brain regions as well as in the SCN (Itri and Colwell, 2003; Cunha-Reis et al., 2004; Hermes et al., 2009; Korkmaz et al., 2010). Thus, VIP deficient animals might not be a suitable model to test the endogenous role of GABA in the SCN network. In addition, the discrepancy between the data is an indicator of multifaceted role of GABA in SCN synchronization. Recently it has been shown that GABA together with VIP, contributes to resynchronization of the SCN network when individual cells are in anti-phase and are not under steady state conditions (Evans et al., 2013).

SCN network organization

Since the maximal activity of most of SCN neurons occurs around midday and only a small population of neurons become active during the night, a sinusoidal pattern of SCN multiunit activity is seen at the ensemble level (Schaap et al., 2003a). The sinusoidal waveform of the multiunit SCN electrical activity can change under different conditions. The degree of the synchronization between SCN neurons determines the distribution of individual neuronal activity pattern and the output of the SCN (Vanderleest et al., 2007; Bodenstein et al., 2012). A wider phase distribution among neurons for instance, results in a broader peak width in the sinusoidal waveform and vice versa; a more compressed distribution of neuronal phases generates a narrower sinusoidal electrical waveform (**Fig. 4**). The ensemble electrical output is different, more stable, more robust and more precise than single cell outputs (Welsh et al., 2010). For instance in aging the SCN network output is more robust than the single cell rhythms (see **chapter 2** for a detailed introduction about aged SCN network). Therefore the network seems to compensate for deficits at the single cell level.

The significance of the network is also manifested by the adjustment of the SCN to seasonal rhythms. In seasonal adaptation, plasticity in the phase relationship between the SCN cells is responsible for changes in the amplitude and waveform of multiunit SCN electrical activity (Rohling et al., 2006; Vanderleest et al., 2007; Brown and Piggins, 2009). The single cell profile is not altered and thus, does not contribute to the changes of the SCN output in seasonality (Vanderleest et al., 2007; Naito et al., 2008). Seasonal encoding therefore is a good model to investigate the network properties of the SCN.

SEASONAL ENCODING AND CIRCADIAN CLOCK

Seasonal changes in the environment have great impact on the physiology of all organisms and their chances to survive. In the winter a severe decrease in temperature and shortage of food urge the organisms to develop winter specific behaviors and physiology e.g. hibernation, migration and timing the mating behavior. During the

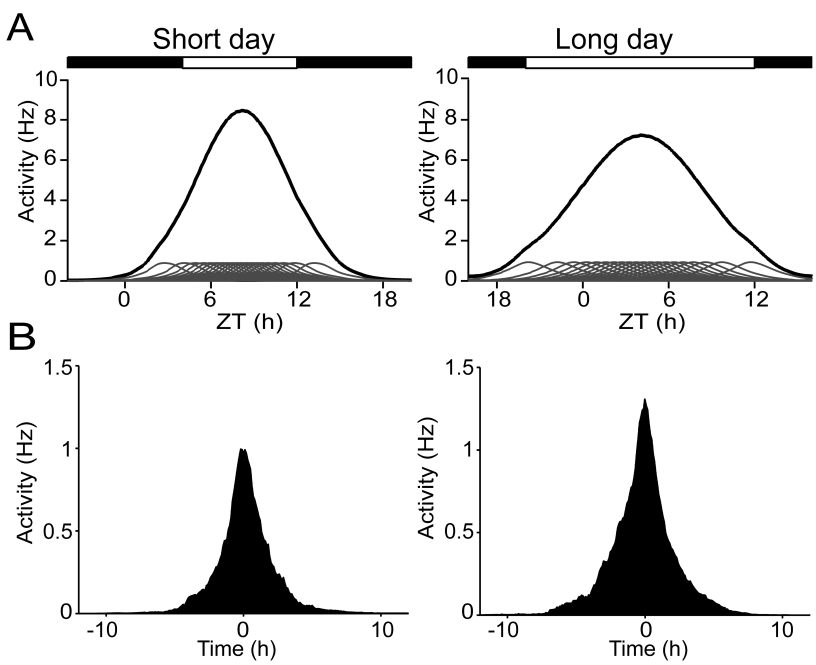

Figure 4. The distribution of single-unit electrical activity patterns determines the sinusoidal waveform of multi-unit electrical activity pattern. (**A**) The multi-unit electrical activity pattern (shown as a thick black line) is compressed in short-day and broadened in long-day photoperiods. This pattern is derived from an ensemble of single-unit activity patterns (gray lines) that are distributed over the 24-h cycle according to a Gaussian distribution. Above the figure, the light/dark schedule for each photoperiod is shown. Adapted from Meijer et al., 2012, progress in brain research. (**B**) Average single-unit activity pattern for short-day and long-day photoperiods is comparable. Adapted from vanderLeest et al., 2007, current biology.

evolution different strategies have been established to predict the environmental annual changes and remain synchronized in physiology and behavior (Paul et al., 2008).

Many animals track the absolute and incremental changes in day length to be able to generate an annual rhythm in their physiology and behavior (Hoffmann, 1979). Even, seasonal reproductive organisms require environmental cues to remain entrained. Hence, the timekeeping mechanisms require photoperiod -or other environmental cues such as food , water, temperature and social cues- to accurately time seasonal events (Reiter, 1974). Photoperiod is the most reliable signal to regulate various physiological functions depending on the time of the year (Johnston et al., 1982; Hoffmann and Illnerova, 1986).

Melatonin

The pineal gland produces melatonin, which has been shown to be important for the seasonal reproduction rhythms in several mammalians (Hardeland et al., 2006; Paul et al., 2008). It also contributes to the regulation of sleep and circadian rhythms and other physiological functions (Hardeland et al., 2006).

Melatonin production is rhythmic with a peak in the night and the duration of the night-time melatonin production is regulated by photoperiod. The pineal gland receives light input through a multi-synaptic neural pathway via the SCN (Moore, 1996). The SCN stimulates norepinephrine release from sympathetic nerves terminals in the pineal gland (Sugden, 1989) and increases the activity of melatonin synthesis enzyme, arylalkylamine N-acetyltransferase (AANAT), at night (Schwartz et al., 2001). The melatonin profile reflects the duration of the night and this signal is therefore well suited to track the changes in photoperiod throughout the seasons (Reiter, 1980).

While melatonin is essential for seasonal reproduction rhythms, it is known that C57BLr6J mice, used in our experiments, do not express melatonin in detectable amounts. Melatonin has been knocked down naturally in these animals due to a point mutation in the gene of melatonin synthesizing enzyme, AANAT (Ebihara et al., 1987; Roseboom et al., 1998). We have shown these animals are able to adapt their physiology and behavior to different photoperiods (Vanderleest et al., 2007). Also, in pinealectomized rats, the SCN exhibits endogenous circadian rhythms that are photoperiod dependent (Sumova et al., 1995). Moreover, no photoperiod response to infusion of melatonin was seen in SCN-lesioned hamsters and only SCN-intact animals were capable to respond to the melatonin infusion (Grosse and Hastings, 1996). Recently, in European hamster the majority of pinealectomized animals were entrained to an accelerated photoperiod schedule which shortens yearly seasonal changes in 6 month (Monecke et al., 2013). These data suggest that melatonin is important for seasonal reproduction but is not the only component which determines the day length to generate seasonal rhythms. Other melatonin independent pathways may also be important for seasonal adaptation. It is clear nevertheless that SCN receives the photoperiodic information that affect both melatonin-dependent and -independent pathways. This indicates a close interaction between the circadian clock and other possible seasonal circuits.

Role of SCN in seasonal encoding

One of the essential functions of the SCN is to entrain an organism to photoperiodic regulation upon seasonal differences. The SCN measures the annual changes in day length and passes this information on to downstream targets. The SCN is able to encode for length of day-light by adjusting the pattern of its electrical activity. Under the influence of long-day and short-day photoperiod, the sinusoidal waveform of the multiunit SCN electrical activity changes both *in vivo* and *in vitro* (Mrugala et al., 2000a; Vanderleest et al., 2007; Houben et al., 2009). The electrical activity pattern is compressed in short-day and is expanded in long-day photoperiod (**Fig. 4A**). Accordingly, the duration of SCN electrical activity pattern in long summer days (LD16:8h) is about 5 hours longer than in short winter days (LD8:16h light; Mrugala et al., 2000a; Meijer et al., 2010). The resulting electrical output signal regulates the duration of daily behavioral activity (α). Accordingly in nocturnal animals, long photoperiod results in a shorter duration of locomotor activity during the night. The photoperiod-induced changes in the SCN waveform and behavioral profile are retained for several days in animals, which are released to constant conditions (Vanderleest et al., 2007; Houben et al., 2009). Thus, the peak width of the SCN electrical activity profile can be used as an endogenous indicator for the length of the day.

Interestingly, in single SCN neurons the differences in day length have no effect on the duration of the electrical activity pattern (**Fig. 4B**) or clock gene expression profile (Vanderleest et al., 2007; Naito et al., 2008). Subsequent studies have shown that the plasticity in phase distribution of single cell rhythms accounts for waveform changes in the electrical activity during photoperiodic adjustment (Rohling et al., 2006; Vanderleest et al., 2007; Brown and Piggins, 2009; **Fig. 4**). Supporting this idea, dissimilar phase distributions between short-day and long-day photoperiod were also observed in clock genes expression such as *Per1*, *2* and *3*, *Cry1* and *2*, *Bmal1*, *Rev-erb* and *Dbp* (Nuesslein-Hildesheim et al., 2000; Sumova et al., 2002; Carr et al., 2003; Johnston et al., 2003; Sumova et al., 2003; Tournier et al., 2003; Johnston et al., 2005; Inagaki et al., 2007; Naito et al., 2008). The expression profiles of all genes are in synchrony during short days. In long days the rostral and caudal neurons of the SCN are desynchronized in *Per1* expression and the peak of the *Per2* is advanced in the caudal compared to the rostral SCN (Hazlerigg et al., 2005; Naito et al., 2008). Moreover, multiple peaks in *Per1* were observed in the rostral SCN of long photoperiod-entrained animals (Inagaki et al., 2007). Therefore, day length encoding in the SCN is likely a neuronal network property rather than a single cell ability. Plasticity in the SCN network may allow broader phase distribution in long-day and narrower phase distribution in short-day photoperiods. The mechanisms underlying this photoperiodic phase adjustments are not completely known. Various mechanisms such as electrical and chemical synapses are responsible for coupling strength within the SCN (Liu and Reppert, 2000; Maywood et al., 2006; Rash et al., 2007; Vosko et al., 2007). VIP is considered a crucial neurotransmitter for photoperiodic adaptation since VIP deficient mice could not encode the photoperiodic alterations after being maintained in constant condition (Lucassen et al., 2012). GABA

in concert with VIP was also shown to be involved in photoperiodic regulation (Evans et al., 2013). More research is required to illuminate the cellular mechanisms, which participate in photoperiodic phase adjustment within the SCN network.

RESEARCH DIRECTIONS

At the cellular level, many properties of the circadian clock neuron are determined by an interaction between membrane events affecting the excitability of the cell and the core molecular clock components (Colwell, 2011). Although little is known about the mechanisms linking the membrane excitability and the regulation of gene expression in SCN neurons, K^+ channels and intracellular Ca^{2+} are among the key players. Many neuronal K^+ channels have an essential impact on the regulation of the action potential frequency and waveform, which in turn affects the amount of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels. Intracellular free Ca^{2+} is an important second messenger, plays a pivotal role in many signaling pathways and is also known to modulate gene transcription in neurons (Berridge, 2012). The study of K^+ currents in the SCN neurons as the central theme of this thesis therefore probes one of the key components of the interdependence between membrane excitability and the molecular clock. The cellular and network properties of the circadian system are altered by aging or in different photoperiods, and modifications of K^+ currents may contribute to these changes. Thus, investigating the functional alterations of K^+ currents in SCN cells caused by aging and photoperiod, will enhance our understanding of the plasticity within the SCN network and its limits.

Aging and exposure of mice to long days affect the circadian clock in similar ways. Both will lead to a circadian phenotype showing (i) a decreased phase-shifting capacity, (ii) a compressed duration of behavioral activity, (iii) wide phase distribution of electrical activity patterns within the SCN network and (iv) a reduced amplitude of the rhythm of the ensemble electrical activity. It is therefore, reasonable to compare the cellular properties of SCN neurons in aged and long-day phenotypes in search for potential common mechanisms.

First, our current understanding of the consequence of aging on circadian clock function in the SCN is reviewed in **chapter 2**. In this chapter the impact of aging on different levels – from the organism to the molecular mechanisms - of the circadian system is discussed. Evidence is reviewed suggesting that even a partially functional neuronal network within the SCN can compensate for more severe deficits on the cellular level. The potential mechanisms for age-related cellular clock dysfunction are presented in a model, revealing the need for more studies to identify the important cellular clock components affected by age. Therefore, in the following two chapters (**chapters 3** and **4**), cellular and network properties of the SCN in the aged circadian system were investigated.

In **chapter 3**, I studied the influence of aging on different levels of the circadian system to determine the contribution of the different clock components to the aged phenotype observed in the behavior. The development of the behavioral aged phenotype was first

determined in a longitudinal study using locomotor activity recordings, from which the period length, the activity duration and the fragmentation of the rest-activity pattern were analyzed. Next, multiunit recordings of SCN slices were performed in young (3-6 month old) and old mice (> 24 month) to determine the impact of age on the electrical output of the SCN. Subpopulation analysis was performed on these MUA data to study the change in neuronal network synchronization at different ages. Finally, I recorded single SCN neurons using patch-clamp techniques to determine the effect of aging on voltage-dependent K^+ currents and neuronal excitability.

Subsequently, one example of the impact of the aging on the dependency of an ion channel and a second messenger is discussed in **chapter 4**. The correlation between a Ca^{2+}-dependent K^+ current (BK) and the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was studied in SCN neurons of mice older than 2 years and compared to a young control group. I used perforated-patch recordings to avoid washout of intracellular Ca^{2+} which could otherwise influence BK current activity. For the measurements of $[Ca^{2+}]_i$ the Ca^{2+}-sensitive dye Fura-2 was used, which enabled quantitative determination of $[Ca^{2+}]_i$.

One of the best models for SCN network plasticity is the photoperiodic phase adjustment within the SCN. It has been suggested that different transmitters, such as GABA and VIP, are involved in adaptation of the phase distribution of the electrical activity profiles to the annual photoperiodic changes. The aim of **chapter 5** was to describe the impact of photoperiod on cellular properties within the SCN. In this chapter, I explore whether and how the single cell properties within the SCN are affected by different photoperiods. In particular I measured the effect of long-day (16h) and short-day (8h) photoperiods on passive membrane properties, neuronal excitability and FDR current activity. A potentially distinct function of the FDR current in photoperiodic entrainment is to facilitate the synchronization between dorsomedial and ventrolateral regions of the SCN. Modification in FDR current may then contribute to various phase distributions under different conditions.

One approach to investigate mechanisms involved in photoperiod-induced phase distribution is to measure the impact of different day length on important neurotransmitter systems known to be involved in synchronization within the SCN network. The most abundant neurotransmitter in the SCN is GABA, and in **chapter 6** I studied the effect of different photoperiods on GABAergic signaling in the SCN. These studies were performed using a combination of whole-cell and perforated-patch recordings as well as Ca^{2+} imaging techniques. First, GABAergic synaptic currents and potentials were recorded in SCN neurons from mice adapted to long-day or short-day photoperiod. Next, excitatory and inhibitory responses to GABA application were recorded as $[Ca^{2+}]_i$ transients and their ratio determined for the different photoperiods. Finally, the GABA equilibrium potential was measured to support the data from Ca^{2+} recordings. This approach aimed at the question if photoperiod can modify a fundamental property of many brain networks - the excitatory to inhibitory balance – in the SCN, which may form the basis for photoperiod induces changes in phase distribution within the SCN neuronal network.

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