A balanced clock: network plasticity in the central mammalian clock

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GENERAL INTRODUCTION

From the earliest emergence of life, organisms have been exposed to a dynamic and constantly changing environment. Through evolutionary processes, species were able to adapt to these changes in order to survive and reproduce. One environmental factor that has hardly changed in time is the rotation of the earth around its axis and its annual orbit around the sun, generating daily and seasonal cycles, respectively. These rather constant environmental time cues in the geophysical environment allowed organisms to develop a clock that could time important events, like feeding and reproduction. Circadian timing mechanisms have a deep evolutionary history, first arising in prokaryotes such as cyanobacteria and (co-)evolved in almost all living species. These timing mechanisms comprise of endogenous circadian rhythms as well as an adaptive, time-measuring system.

Until the 18th century it was generally assumed that all behavioral rhythms were caused by environmental cues, like day light, temperature, and the magnetic field of the earth. In 1729 Jean-Jacques de Mairan observed, for the first time, an internal timekeeping mechanism in the mimosa plant. The plant followed the sunlight with its branches and leaves throughout the day. When de Mairan transferred the plant to constant darkness, the rhythmic movement of the plant continued. This surprising finding showed that external light is not necessary to maintain circadian rhythmicity (de Mairan, 1729). Two hundred years later, Maynard Johnson presented observations that mice showed a sustained activity rhythm with a period that deviated slightly from 24 hours when placed in constant darkness (Johnson, 1926). Johnson was the first researcher who established that mice are able to intrinsically regulate a rhythmicity of about 24 h. Since then, many researchers documented that organisms, ranging from unicellular to mammals, exhibit endogenous circadian rhythms (Pittendrigh, 1960; Cohen & Golden, 2015).

1. CIRCADIAN RHYTHMS

1.1. Entrainment

As mentioned above, without external timing cues circadian rhythms in behavior persist, thereby demonstrating the existence of an endogenous timing system. The period of this internal rhythm deviates slightly between individuals, and also between species, and is approximately one day (hence "circa"-"dian"). For instance, when a mouse is placed in a cage equipped with a running wheel in constant darkness, the wheel running rhythm will remain, but the mouse will start and stop running a few minutes earlier every cycle. This "free-running" rhythm displays the internal period of the mouse, which is often shorter than 24 h and referred to as "tau" (Figure 1). Despite their endogenous nature, circadian rhythms are responsive to light and darkness. Under natural conditions there is adaptation of the organisms circadian clock to the environmental cycle, thus matching internal time to the solar time. This is a key property of the circadian system and is known as "entrainment" (Pittendrigh & Daan, 1976), for review: (Golombek & Rosenstein, 2010).

The adaptive characteristic of the circadian system enables organisms to survive under changing conditions by anticipating periodic events, such as the availability of food. Light is the most important timing signal ("zeitgeber") for keeping the internal circadian rhythms synchronized to the environmental 24 h cycle. There are, however, additional non-photic

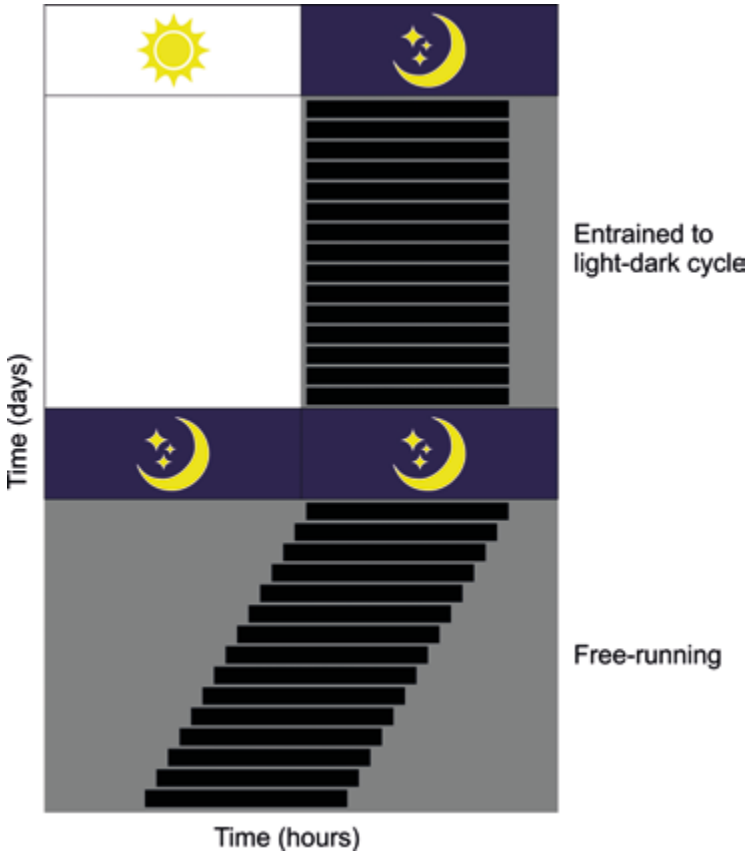

Figure 1. Circadian rhythm in behavioral activity of the mouse. Schematic representation of an actogram of the behavioral wheel running activity of a mouse. Consecutive days are plotted below each other. The black bars represent wheel running activity. In the upper half (first 15 days), the mouse is entrained to a light-dark cycle of LD 12:12. In the lower half (days 16-30), the mouse is placed in constant darkness and starts its "free-running" behavioral activity ~0.5 h earlier every day due to its internal period of 23.5 h.

zeitgebers, like environmental temperature, feeding cycles, and behavioral activity that provide input to the circadian clocks and can modulate its function (Hughes & Piggins, 2012; Lewis *et al.*, 2020). In contrast to the photic stimuli, non-photic zeitgebers generally have the opposite effect on the phase of the circadian clock. The generation of a timing signal by the mammalian clock is thus a complex interplay between photic and non-photic stimuli (Challet & Pevet, 2003).

1.2. Molecular clock

Circadian rhythmicity is driven by numerous clock genes that are expressed with a period of about 24 h. In 2017, the award of the Nobel Prize in Physiology or Medicine was awarded to Jeffrey C. Hall, Michael Rosbash, and Michael W. Young for their "discoveries of molecular mechanisms controlling the circadian rhythm" which is an acknowledgement of the fundamental importance

of circadian clocks (Callaway & Ledford, 2017). These three investigators unraveled the molecular pathway that controls the internal circadian rhythms in fruit flies (Bargiello *et al.*, 1984; Zehring *et al.*, 1984; Price *et al.*, 1998). Since that discovery, many studies were carried out to also identify the mammalian version of these genes (Shearman *et al.*, 1997; Gekakis *et al.*, 1998; Sangoram *et al.*, 1998). The unifying principle of cellular circadian timekeeping, in all organisms investigated, is based on self-sustained transcriptional-translational feedback loops (TTFLs).

In mammalian cells, the main loop starts when CLOCK proteins binds to BMAL1 proteins to form a dimer that binds to the E-box regions in the promotor of clock genes and thereby drive the daytime transcription of period (*Per1, Per2,* and *Per3*) and cryptochrome (*Cry1* and *Cry2*) genes (Buhr & Takahashi, 2013). Over the course of the day, the products of these genes, PER and CRY, accumulate and form dimers. These dimers translocate back into the nucleus of the cell where they suppress CLOCK-BMAL1 activity at their own E-boxes. During the night, subsequent degradation of PER and CRY allows the CLOCK-BMAL1 dimers to eventually start the cycle again. This core TTFL is stabilized and enhanced by additional negative and positive feedback loops. For instance, CLOCK and BMAL1 drive E-box mediated circadian expression of the nuclear receptors RORα, REV-ERBα, and REV-ERBβ which in turn act via REV response element (RRE) sequences to activate and suppress BMAL1 transcription, respectively (Lowrey & Takahashi, 2004; Hastings *et al.*, 2018). In addition, oscillations in cytosolic signaling molecules, like cAMP and Ca^{2+}, interact with the different TTFLs to further stabilize and tune the cellular molecular clocks (Hastings *et al.*, 2008). The TTFL is highly conserved across species and present in almost all mammalian cell types providing local timing systems in most tissues (Reppert & Weaver, 2001). However, these peripheral clocks are like instruments in an orchestra, which are directed by a conductor. In mammals, this conductor is the central circadian clock which is located in the brain.

2. CENTRAL CIRCADIAN CLOCK IN MAMMALS: THE SUPRACHIASMATIC NUCLEUS

First evidence for the location of the mammalian biological clock was found independently in 1972 by two laboratories. Both research groups lesioned the brain region in the anterior hypothalamus, directly above the optic chiasm; the suprachiasmatic nucleus (SCN), which caused the disappearance of behavioral rhythms in drinking and running activity (Stephan & Zucker, 1972) and in corticosterone production by the adrenal gland (Moore & Eichler, 1972). Although these studies demonstrated that an intact SCN was necessary for the expression of circadian rhythms, they did not fully prove that the SCN was the location of the central mammalian pacemaker. Actual evidence came a few years later when another important study performed in vivo electrophysiological recordings in the SCN. The electrical activity in the SCN appeared rhythmic with high activity during the day and low during the night while surrounding tissue was oscillating in antiphase. When the SCN was completely isolated from the rest of the tissue, the surrounding tissue lost their rhythmicity, while the SCN remained rhythmic (Inouye & Kawamura, 1979). After this pivotal study that drove neurophysiological studies in the SCN, three different research groups demonstrated in 1982 that the SCN could be isolated in brain slices and that the rhythmicity in electrical activity remained

(Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata *et al.*, 1982). These findings indicated that the SCN does not require rhythmic input, but remains oscillating endogenously, also when kept in constant conditions. The most conclusive evidence that the SCN are indeed the site of the mammalian circadian pacemaker came more than a decade later by transplantation studies in which restoration of circadian rhythmicity was observed after transplantation of fetal SCN tissue in arrhythmic hamsters whose own SCN had been ablated. Moreover, using tau mutant hamster as donors, the free running period length of the host wild type hamster (ca 24 h) was changed to the one of the donor (20 h) (Ralph *et al.*, 1990; Silver *et al.*, 1996).

2.1. Anatomical and functional organization of the SCN

The SCN is located in the anterior hypothalamus, directly above the optic chiasm and bilateral to the third ventricle, and consists of two connected nuclei. Each nucleus consists of about 10.000 neurons and is subdivided into a "core" and "shell" based on the distribution of peptide phenotypes and efferent and afferent connectivity (Moore, 1996).

The ventrolateral core contains vasoactive intestinal polypeptide (VIP) and gastrin releasing peptide (GRP) and receives direct light input through the retinohypothalamic tract (RHT). The dorsomedial shell contains a large number of argenine vasopressin (AVP) producing cells and receives input from the SCN core, and limbic, hypothalamic, and brainstem nuclei (Abrahamson & Moore, 2001). Additionally, almost all SCN neurons express γ-aminobutyric acid (GABA) receptors. Anatomical studies generally support a neuropeptide-based classification of the SCN subtypes (Leak *et al.*, 1999; Abrahamson & Moore, 2001; Masumoto *et al.*, 2006). However, the functional heterogeneity within the SCN network is more complicated (Morin, 2007). New SCN cell types were discovered by studying single cell transcriptional heterogeneity, which led to new interpretations of the functional variability and connectivity of SCN neurons (Park *et al.*, 2016). The majority of VIP and AVP neurons co-express the neuropeptide neuromedin S (NMS), which has shown to dictate circadian period length and is required for the generation of behavioral rhythms. The detection of NMS cells as a population of pacemaking neurons allows for better targeting of treatment of circadian dysfunction (Lee *et al.*, 2015).

The SCN exhibits precise and high-amplitude rhythms in electrical activity and communicates this time information to the rest of the brain and body. Regardless of whether an organism is diurnal (day-active) or nocturnal (night-active), the ensemble electrical discharge in the SCN is high during the day and low during the night (Meijer *et al.*, 1997; Caldelas *et al.*, 2003). When the retina is exposed to light, the membrane potential of retinorecipient SCN cells are sufficiently depolarized to generate action potentials. Apart from these light-induced potential changes, SCN cells exhibit circadian rhythms in spontaneous firing frequencies (Webb *et al.*, 2009). The neurons of the SCN have this unique property of generating spontaneous action potentials for about 4-6 hours each day, usually during the daytime (Schaap *et al.*, 2003; Brown *et al.*, 2006; VanderLeest *et al.*, 2007). Several currents account for this daily rhythm in action potential generation, like persistent Na^+ currents, L-type Ca^{2+} currents, hyperpolarization-activated currents (I_H), large-conductance Ca^{2+} activated K^+ (BK) currents, and fast delayed rectifier (FDR) K^+ currents (Brown & Piggins, 2007; Colwell, 2011; Harvey *et al.*, 2020). The electrical activity of the individual SCN neurons will together

generate an ensemble electrical signal, which will be corrected in phase by the retinorecipient cells in order to align the SCN output signal to the external zeitgeber cycles.

2.2. The SCN as a neuronal network

When dispersed in culture, individual SCN neurons can maintain cell-autonomous circadian cycles in gene expression, intracellular calcium concentration, and neuronal firing rate (Noguchi *et al.*, 2017). However, these cells often form only erratic networks. As a result the rhythms are more sloppy and the isolated neurons show independent free-running rhythms with a large variation in period length (Welsh *et al.*, 1995; Herzog *et al.*, 2004). When the network maintains more intact, like in organotypic slices (Tominaga *et al.*, 1994), the ensemble rhythmic output of the SCN is more precise and the phases of individual cells are better synchronized. Communication and synchronization between SCN cells are key for generating a coherent, stable, and high amplitude output rhythm and enable the circadian system to adapt to environmental changes. Some SCN neurons are electrically coupled by gap junctions (Colwell, 2000; Long *et al.*, 2005), but all cells communicate via transmissions of action potentials after which neurochemicals like VIP and GABA are released . In addition, neuroendocrine signaling, i.e. the release of neurohormones like GRP and AVP, is an important communication factor in the SCN (Mohawk & Takahashi, 2011; Maywood, 2018; Astiz *et al.*, 2019).

VIP signaling is critically important for synchronization among the SCN neurons as loss of VIP (VIP/PHI$^{-/-}$) or the gene encoding its receptor in the SCN, VPAC2 (*Vipr2*$^{-/-}$), leads to weakened circadian rhythmicity in electrical activity and amplitude, gene expression, and metabolism (Vosko *et al.*, 2007). Moreover, the SCN loses its synchrony to environmental light cues and behavioral rhythms in VIP/PHI$^{-/-}$ and *Vipr2*$^{-/-}$ mice are disrupted (Harmar *et al.*, 2002; Colwell *et al.*, 2003; Aton *et al.*, 2005; Maywood *et al.*, 2006; Brown *et al.*, 2007; Sheward *et al.*, 2007; Lucassen *et al.*, 2012).

Almost all SCN neurons contain GABA as a neurotransmitter (Moore & Speh, 1993; Abrahamson & Moore, 2001; Belenky *et al.*, 2008), but in contrast to the advanced knowledge of the role of VIP in SCN synchronization, the role of GABAergic signaling in the SCN is still subject of debate (Ono *et al.*, 2018). GABA was initially reported to synchronize rhythms in electrical activity in dispersed clock cells by daily application of GABA (Liu & Reppert, 2000). Moreover, GABAergic signaling proved to be necessary for coupling between the ventral and dorsal part of the SCN after a shift in the light-dark cycle (Albus *et al.*, 2005). But, on the other hand, GABA antagonist have shown to increase the amplitude of PER2::LUC rhythms by reducing the phase distribution of the individual SCN cells (Aton *et al.*, 2006). Additionally, inhibition of $GABA_A$ signaling using gabazine decreased period variability in cultured SCN slices, suggesting that GABA has a rather destabilizing effect on the SCN rhythms (Freeman *et al.*, 2013). Other work suggested that the role of GABA in synchronization depends on the state of the SCN neuronal network. $GABA_A$ signaling promoted re-synchronization when the dorsal and ventral SCN rhythms were 6-12 h apart, but opposed synchronization when the SCN was in a steady-state (Evans *et al.*, 2013).

It should be noted that the role of GABA in coupling might depend on the polarity of the neuronal response, as GABAergic signaling can be inhibitory as well as excitatory in the SCN (Wagner *et al.*, 1997). Several studies have found that the polarity of the GABAergic response depends on

the circadian phase, regional localization, and even the length of the day (i.e. photoperiod) (De Jeu & Pennartz, 2002; Albus *et al.*, 2005; Choi *et al.*, 2008; Farajnia *et al.*, 2014b).

The GABAergic response is based on the chloride (Cl^-) flow across the cell membrane through a GABA-gated chloride channel. The direction of the Cl^- flow (in or out of the cell), and thus the polarity of the GABAergic signal, depends in part on the intracellular concentration of chloride ($[Cl^-]_i$) and the relationship between the chloride equilibrium potential (E_{Cl}^-) and the membrane potential (V_m) (Kaila, 1994; Ben-Ari, 2002). Low levels of $[Cl^-]_i$ lead to a Cl^- influx through the GABA receptor mediated channel and subsequent hyperpolarization of the membrane potential, and thus to an inhibitory action of GABA (Figure 2A). Under certain conditions, however, $[Cl^-]_i$ can be higher and E_{Cl}^- can be more depolarized relative to the resting membrane potential. This causes an outward flow of Cl^- when the GABA channel opens and leads consequently to depolarization and excitatory action of GABA (Figure 2B). Different classes of cation-chloride-cotransporters (CCCs) regulate $[Cl^-]_i$. The SCN expresses at least two of the CCC transporters that regulate $[Cl^-]_i$ and determine the chloride equilibrium potential (Choi *et al.*, 2008; Belenky *et al.*, 2010); the Na^+-K^+-$2Cl^-$ cotransporter 1 (NKCC1) transporting Cl^- out of the cell and the K^+-Cl^- cotransporter 2 (KCC2) which activation leads to Cl^- accumulation in the cell (Blaesse *et al.*, 2009; Rohr *et al.*, 2019). In the immature brain, the E_{Cl}^- and $[Cl^-]_i$ are high due to early expression of NKCC1, leading to excitatory actions of GABA. During development there is a switch in the expression of NKCC1 and KCC2 and in mature neurons higher expression levels of KCC2 lead to Cl^- influx and hyperpolarization, upon $GABA_A$ receptor activation (Ben-Ari, 2002). However, even in the mature brain, GABAergic depolarization and excitation has been reported for multiple brain areas, including the SCN (Chung, 2012). Recently, it was demonstrated that indeed a switch in regulation of KCC2 and NKCC1 expression corresponds with plasticity in $GABA_A$ signaling in the SCN (Rohr *et al.*, 2019). In the SCN, the KCCs are the primary regulators of $[Cl^-]_i$, whereas the chloride uptaker NKCC1 contributes moderately to regulating $[Cl^-]_i$ (Klett & Allen, 2017). The polarity of the GABAergic response, being excitatory or inhibitory, consequently determines the E/I balance in the SCN. The right balance between excitation and inhibition is crucial for proper information processing and thus brain functioning (Zhou & Yu, 2018). Since GABA is the principle neurotransmitter in the SCN, it is also the main contributor to the E/I balance. Both GABA and the E/I balance are thought to play important roles in synchronization and communication within the SCN network (Farajnia *et al.*, 2014b; Myung *et al.*, 2015; Kim *et al.*, 2019; Rohr *et al.*, 2019) which are needed for proper adjustment to external light-dark cycles.

2.3. Entrainment of circadian rhythms by the SCN

Communication and coupling within the SCN network are important to generate a robust output signal to the periphery, but also to synchronize the SCN to the environmental conditions. Synchronizing the internal timing to the outside world is achieved by "phase shifting" actions of the SCN. SCN cells can adjust the phase of their circadian rhythm in response to zeitgeber signals, of which light is the most important one. This phase shifting capacity of the SCN is based on a time-dependent responsiveness of the network to light. When animals are kept under constant conditions, like constant darkness, and are exposed to a short light pulse, they will shift their rhythms

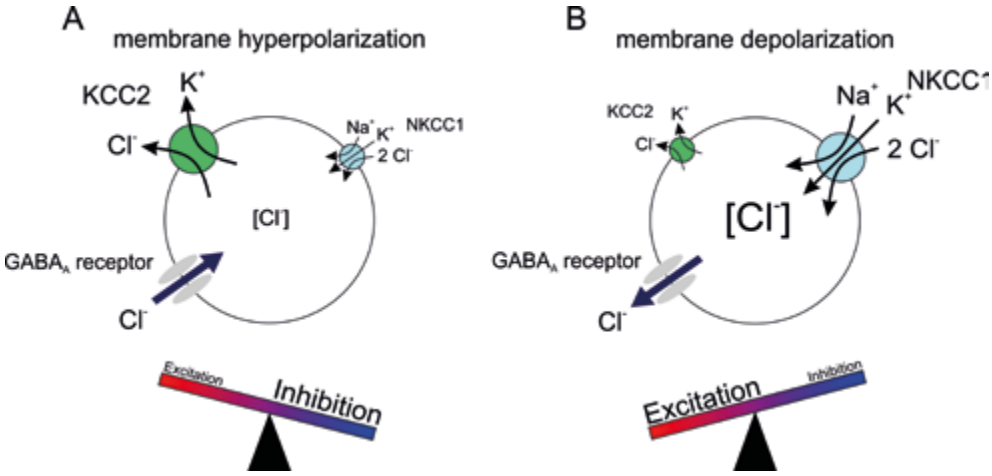

Figure 2. GABAergic activity in SCN cells. A. When there is relatively more expression of the Cl^- extruder KCC2, the intracellular Cl^- concentration will be low. If the GABA receptor is activated, the GABA-gated Cl^- channel will open and there will be an inward flow of the negatively charged Cl^- ions. This will hyperpolarize the membrane and result in an inhibitory action. B. In case there is relatively more NKCC1 expression, the intracellular Cl^- levels will be high. When the GABA-gated Cl^- channel opens, Cl^- flows out of the cell causing a depolarization of the membrane potential and consequently an excitatory response.

depending on the timing of the light pulse. Light pulses presented during the "subjected" early night will phase delay the rhythms, whereas at the end of the "subjected" night, the light exposure will induce phase advances (Daan & Pittendrigh, 1976). This phase dependent responsiveness of the SCN is essential for proper entrainment to the surrounding environment and a common response property of all living organisms. Phase shifting capacity is diminished when the SCN is in a more desynchronized state with low amplitude (vanderLeest *et al.*, 2009), emphasizing the importance of coupling within the SCN.

2.4. Encoding day length by the SCN

As described above, the SCN uses coupling mechanisms, on the one hand, to synchronize the rhythms of the individual cells resulting in a coherent signal to the periphery, and on the other hand, to adjust the phase of the SCN rhythm to the environmental conditions. The rotation of the earth on its axis causes day-night changes in light exposure. In addition, the SCN encodes for seasonal changes due to the earth's annual revolution around the sun. Seasonal changes in the environment, like fluctuations in photoperiod, light intensity, temperature, and food availability, have great impact on the physiology of all organisms. Although daily timekeeping is a property at the level of the SCN neurons, the SCN network is needed to measure the annual changes in day length.

Ex vivo electrophysiological recordings from single cells shows that the activity patterns of single SCN cells do not change with photoperiod. As under a 12 h photoperiod, individual SCN cells

are electrically active for a relatively short amount of time (4 – 6 h) when exposed to a short or long photoperiod (Schaap *et al.*, 2003; Brown & Piggins, 2009). The SCN is able to encode for different photoperiods by adjusting waveform changes of the ensemble electrical activity. The distribution of single-cell patterns of electrical activity are compressed, meaning that the peak in their activity is clustered, when mice are entrained to a short day photoperiod (Figure 3A), whereas the activity patterns are more dispersed when exposed to a long day photoperiod (Figure 3B). The change in distribution of phases within the SCN causes the sinusoidal waveform of the ensemble electrical activity to change, both in vivo as ex vivo, resulting in a more narrow peak width with high amplitude in short days and a wider peak width with low amplitude in long days (Mrugala *et al.*, 2000; VanderLeest *et al.*, 2007). Accordingly, the phase relationship between the SCN neurons determines the multiunit electrical activity pattern, and the ensemble waveform change is responsible for photoperiodic encoding in the SCN.

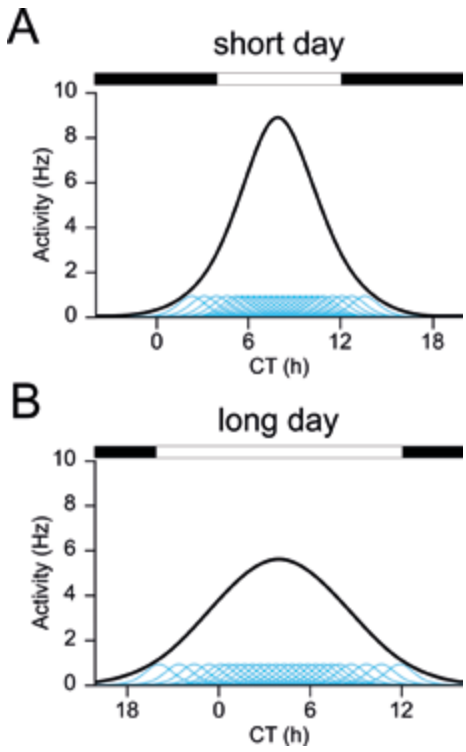

Figure 3. The distribution of single cell electrical activity patterns determine the multiunit electrical activity waveform in long and short photoperiod. The shape of the multiunit electrical activity waveform (shown as a thick black line) is derived from the summation of single-unit activity patterns (blue lines) that are distributed over the 24 h cycle according to a Gaussian distribution. This distribution is narrow in a short day (LD 8:16) photoperiod (A) and broad in a long day (LD 16:8) photoperiod (B). Above the figures, the light-dark schedule for each photoperiod is shown. White bars represent daytime, and black bars represent nighttime. Adapted from Meijer *et al.*, 2012

In vivo electrophysiological recordings in freely moving mice show that the transitions in behavior – i.e. the onset and offset of behavioral activity – occur at half maximal levels of the SCN's electrical activity (Houben *et al.*, 2009). When mice are exposed to long and short day photoperiods, the electrical output signal regulates the duration of behavioral activity in a similar manner. Indeed, exposure to long and short photoperiods has significant effects on the behavioral patterns in wheel-running activity in mice, with compressed and expanded durations of the activity period, respectively (Refinetti, 2002; VanderLeest *et al.*, 2007). Thus, the expanded and compressed waveform in multiunit electrical activity correspond to compressed and expanded durations of nocturnal behavioral activity, under a long and short day photoperiod, respectively (Houben *et al.*, 2009). The photoperiod-induced changes in behavioral activity and in the waveform of electrical activity retain for several days in rodents, after release into constant conditions. In other words, the SCN has a "memory" for photoperiod. This memory is of importance when using ex vivo methods to study photoperiodic encoding in the SCN tissue.

Similar to the electrophysiological studies, molecular studies have shown that differences in day length do not influence single-cell gene expression profiles, but rather affect the SCN at the network level (Naito *et al.*, 2008). The phase distribution of several clock genes, like *Per1* and *2*, *Cry1* and *2*, *Bmal1*, *Rev-erb* and *Dbp*, changes when exposed to different photoperiods (Sumová *et al.*, 2002; Sumová *et al.*, 2003; Hazlerigg *et al.*, 2005; Johnston *et al.*, 2005; Inagaki *et al.*, 2007; Naito *et al.*, 2008; Sosniyenko *et al.*, 2009; Buijink *et al.*, 2016).

Accordingly, photoperiodic encoding by the SCN is a neuronal network property, rather than a capability of the single neurons. Plasticity, coupling strength, and communication within the SCN network is therefore crucial for proper seasonal encoding and allows for changes in phase distribution. However, the exact mechanisms responsible for photoperiodic phase alterations are still not completely understood. As discussed earlier this chapter, several neurotransmitters, like VIP and GABA, are good candidates for coupling mechanisms within the SCN (Maywood *et al.*, 2006; Vosko *et al.*, 2007; Rohr *et al.*, 2019) and thus also for seasonal encoding. When neurons are desynchronized in phase, like under an extreme (long) photoperiod, the SCN neurons resynchronize via coupling processes mediated by both VIP and $GABA_A$ signaling (Evans *et al.*, 2013). Accordingly, VIP knock out mice lose the ability to adapt to either long or short photoperiods (Lucassen *et al.*, 2012). The polarity of GABAergic responses also changes under the influences of different day lengths, with more GABAergic inhibition in SCN slices of mice exposed to short photoperiod and more GABAergic excitation under long photoperiod. These results suggest that the mechanisms that contribute to the degree of synchronization might partly depend on the E/I ratio (Farajnia *et al.*, 2014b).

3. AGING

The degree of synchronization and the strength of intercellular coupling may, potentially irreversibly, be influenced by aging or disease. Aging inevitably affects many aspects of (brain) physiology and behavior, including the circadian system (Buijink & Michel, 2020). At the behavioral level, age-related changes often lead to a reduction in behavioral activity levels and fragmented

sleep-wake rhythms in both humans and animal models (Valentinuzzi *et al.*, 1997; Dijk & Duffy, 1999; Hofman & Swaab, 2006; Farajnia *et al.*, 2012). Older mice show longer latencies to re-entrain their locomotor activity rhythms after phase shifts (Biello, 2009). Exposure to repeated abrupt changes in the phase of the light-dark cycle can even lead to higher mortality rate in old mice (Davidson *et al.*, 2006). These behavioral deficits are likely the consequence of a weakened timing signal generated by the SCN (Oster *et al.*, 2003; Farajnia *et al.*, 2014a).

Both in vivo and ex vivo studies showed a significant reduction in the amplitude of the ensemble electrical activity rhythm of the aged SCN (Satinoff *et al.*, 1993; Watanabe *et al.*, 1995; Nakamura *et al.*, 2011; Farajnia *et al.*, 2012). Surprisingly, there is no, or only limited, age-related neuronal cell loss in the SCN (Roozendaal *et al.*, 1987; Miller *et al.*, 1989; Fernandez *et al.*, 2021). The reduction in output signal is more likely the consequence of decreased synchronization within the SCN network (Watanabe *et al.*, 1995; Farajnia *et al.*, 2012). In addition, there are studies that showed significant deficits in circadian modulation of the physiology of individual SCN neurons (Aujard *et al.*, 2001; Farajnia *et al.*, 2012).

Ex vivo electrophysiological recordings showed changes in the phase distribution of single cell neuronal activity rhythms in SCN slices of aged mice. Subpopulations of neurons had their peak in electrical activity during the middle of the night, so in antiphase to the peak in electrical activity of most subpopulations. In contrast, SCN activity in young control slices clustered around the middle of the day (Farajnia *et al.*, 2012). These anti-phase oscillations cause reduced synchronization within the network which is likely responsible for the diminished amplitude of the SCN output signal.

The neurotransmitters VIP and GABA contribute to synchronization mechanisms in the SCN network and are both affected by aging. The number of VIP expressing neurons within the SCN declined in very old rats (Chee *et al.*, 1988) and humans (Zhou *et al.*, 1995). Also, the VIP and VPAC2 receptor mRNA expression rhythm was altered during the aging process (Kawakami *et al.*, 1997; Krajnak *et al.*, 1998; Kalló *et al.*, 2004). The number of GABAergic synaptic terminals in the SCN are diminished by 26% due to aging (Palomba *et al.*, 2008) and GABAergic postsynaptic currents are reduced in frequency and amplitude (Nygard *et al.*, 2005; Farajnia *et al.*, 2012). These age-associated alterations in neurotransmitter functionality could account for the reduced phase coherence between SCN neurons.

Besides the changes at the network level, there is also evidence for age-related deteriorations at the level of the single cell (Aujard *et al.*, 2001; Farajnia *et al.*, 2012). Among these cellular deficits are changes in membrane properties of SCN neurons. Aging was shown to diminish the circadian regulation and function in a number of ion channels. For instance, the FDR channel and the transient K^+ channel exhibited a loss in circadian rhythmicity in channel activity in old mice, which could contribute to a change in firing frequency (Farajnia *et al.*, 2012). Also, the circadian rhythms in the BK current are impaired in SCN neurons during aging which caused significant changes in action potential waveform (Farajnia *et al.*, 2015). These age-related changes in SCN ion channels and currents are likely to contribute to the lack of circadian modulation of membrane potential and membrane conductance observed in the aged SCN neurons (Farajnia *et al.*, 2012). The cellular deficits possibly lead to dysregulation of SCN calcium (Ca^{2+}) rhythms, since the levels of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) were found to be reversed in aged mice, with high $[Ca^{2+}]_i$ at night and

normal levels during the day, when compared to the SCN of young mice (Farajnia *et al.*, 2015). Ca^{2+} ions are important for regulating molecular mechanisms of rhythm generation (Lundkvist *et al.*, 2005) and play an important role in photic responses (Irwin & Allen, 2007). A possibly disrupted Ca^{2+} homeostasis will affect neuronal communication, but also basic rhythm generation, and is therefore an interesting target for restoring age-related clock dysfunction.

4. OUTLINE OF THIS THESIS

The experimental research in this thesis aims to gain more understanding of how the SCN network is organized and what is needed for network changes. More specifically, this work focused on the potential role of GABA and the GABAeric E/I balance in SCN network plasticity. Communication and synchronization in the SCN are important for the generation of a strong and coherent output signal. Under certain conditions, like long photoperiod, the phases of the individual SCN cells are more dispersed over the 24 hour cycle as evidenced by measurements of electrical activity and clock gene expression. Aging is also known to affect the network organization of the SCN with deterioration in synchronization among the individual SCN neurons. In this thesis, I present work that contributes to research questions regarding the effect of light exposure and/or aging on several characteristics of SCN network plasticity.

Chapter 2 describes a series of studies that examine whether exposure to light for the full length of the day was needed to achieve cellular reorganization within the SCN network as demonstrated after entrainment to long or short photoperiod. In other words, is reception of the full photoperiod needed for photoperiodic encoding in the SCN, or does exposure to short light pulses at the beginning and end of the day suffice? To examine this, mice were exposed to "skeleton photoperiods" that mimicked long summer days of 16 hours or short winter days of 8 hours. Skeleton photoperiods consist of two brief light exposures of 30 minutes that mark the beginning and end of the day. Early studies have shown that nocturnal rodents are very well capable to behaviorally adapt to these skeleton photoperiods (Pittendrigh & Daan, 1976; Stephan, 1983). In **chapter 2** multiple behavioral, cellular, and molecular experiments were conducted to examine the level of SCN network adaptations after entrainment to skeleton photoperiods.

Photoperiodic phase adjustment in the SCN network is a good example of SCN network plasticity. As described in this introduction, previous research has shown that aging affects the circadian clock at different levels, among which the network level. In **chapters 3 and 4** we further investigated the effect of aging on several cellular, network, and behavioral properties of the circadian system. In **chapter 3**, the plasticity of the circadian clock in old mice was examined at the behavioral level by recording activity while mice were exposed to different photoperiods. After the behavioral recordings, measurements of single-cell bioluminescence imaging of the clock gene per2 were made. The aim was to investigate if, and how, the molecular clock adapts to changing day lengths with aging. Measuring the phase distribution of clock gene expression under different conditions allowed to explore the level of plasticity in the old SCN network.

The effect of aging on GABAergic function, the concomitant E/I balance, and on Ca^{2+} homeostasis was investigated in **chapter 4**. During aging, the SCN network becomes less synchronized and

chapter 4 assessed whether the E/I balance could play a potential mechanistic role. To address this question, the polarity of Ca^{2+} transients in response to exogenous GABA stimulation was determined in SCN slices of old (20-24 months) mice and young controls. From these results, the E/I balance was established in the old and young SCN. Moreover, the baseline Ca^{2+} transients provided information on the $[Ca^{2+}]_i$ of old and young SCN neurons and thus on the effect of aging on calcium homeostasis.

The research described in this thesis, and results from other studies, suggest that the balance between GABAergic excitation and inhibition plays a role in synchronization and/or plasticity of the SCN network. However, the exact role or mechanism remains topic for future studies. Pharmacological manipulations of GABAergic signaling, or chloride levels, are important tools in both in vivo and in vitro research regarding the E/I balance. In **chapter 5** experiments with a newly developed blocker – ML077 – of the KCC2 channel were conducted to investigate the efficiency of this blocker and the role of KCC2 in the GABAeric responses in SCN slices. Using calcium imaging, GABA-induced single-cell Ca^{2+} transients were recorded before and after blocking KCC2 with ML077 in SCN slices of mice entrained to different photoperiods. ML077 proved to be able to shift previously GABAergic inhibitory responses to excitatory ones and promises to be a powerful tool in researching the effect of E/I balance on the level of synchronization in the SCN network.

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