A balanced clock: network plasticity in the central mammalian clock

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GENERAL DISCUSSION

The central circadian clock in mammals resides in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus in the brain. The SCN clock is composed of multiple, single-cell oscillators which together generate an output signal, synchronized to the environmental light-dark cycle, and convey this "timing information" to the periphery. The functioning of the SCN as the major pacemaker relies on the communication and coupling between the cells in the network, and on the ability of the network to adjust to environmental cycles. Besides its role as a daily clock, the SCN also serve as a seasonal clock by adjusting the output signal to the duration of light per day (i.e. photoperiod). Seasonal encoding by the SCN is a good example of the functionality of the network – opposed to the individual SCN neurons being autonomous oscillators –, since it is a composite tissue property that relies on the network organization, and amongst others, on the synchronization of single-cell oscillators.

The work in this thesis enhances our understanding of what is needed for this type of network plasticity. Specifically, we focused on the potential role of the neurotransmitter GABA and the GABAergic Excitation/Inhibition (E/I) balance in coupling mechanisms within the SCN network under different conditions, like changing light regimes and aging. We showed for the first time that brief light exposure at the beginning and end of the day can change the function of GABA. We also provided evidence that chloride cotransporter modulation is responsible for light regime induced changes in E/I balance. Moreover, we discovered a shift in E/I balance in the aging central clock that may explain network plasticity. In this chapter I will explain and discuss the implications of these outcomes.

1. E/I BALANCE IN THE BRAIN

The right balance between excitation and inhibition is crucial for proper brain function, because synaptic E/I balance underlies efficient neuronal information processing (Zhou & Yu, 2018). Loss of a tight control over the E/I balance could result in hyperexcitability or excessive quiescence, which in turn cause deficits in information processing (Figure 1) (Kinouchi & Copelli, 2006). The implications of an E/I imbalance can be detrimental. Abnormalities in inhibition or excitation are linked to disorders that occur particularly at the beginning and the end of our life, like several neurodevelopmental as well as neurodegenerative disorders (Gatto & Broadie, 2010; Rissman & Mobley, 2011; Canitano & Pallagrosi, 2017; Bi *et al.*, 2020; Bruining *et al.*, 2020).

Moreover, dysregulation of the E/I balance is correlated to the pathophysiology of migraine and epilepsy (Sgadò *et al.*, 2011; Vecchia & Pietrobon, 2012). The mechanisms by which a balance between excitation and inhibition is established and maintained is still a subject of debate. Physiologically, there are homeostatic and developmental processes that maintain the E/I balance in a narrow range at the level of single cells and at the level of cortical networks over long timescales (Zhou *et al.*, 2014; He *et al.*, 2016). The E/I balance of single cells is determined by the relative contributions of excitatory and inhibitory synaptic currents (Shu *et al.*, 2003; Okun & Lampl, 2008). It is widely accepted that an E/I balance is kept approximately constant and that deviations from the E/I ratio beyond acceptable tolerances result in aberrant hyper- or hypo-activity. However, there is also evidence that small changes of the E/I balance are physiologically relevant. For instance, during

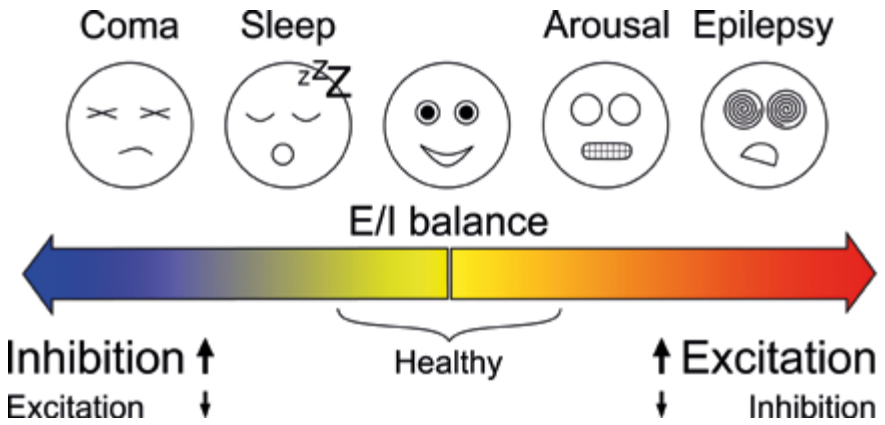

Figure 1. The excitation/inhibition balance. A schematic overview of the balance between excitation and inhibition in the brain. A proper balance between excitation and inhibition is crucial for brain functioning. When the scale turns towards more inhibition, this results in excessive hypo-activity like sleepiness or even a coma. When the scale turns towards too much excitation, this results in hyper-excitability like arousal or even seizures.

sensory stimulation in the auditory cortex, E/I balance can be transiently perturbed, but generally recovers within tens of minutes (Froemke *et al.*, 2007). On a longer timescale, there is a circadian regulation in E/I balance in cortical areas in humans (Chellappa *et al.*, 2016; Ly *et al.*, 2016) and there are indications for seasonal variability (Meyer *et al.*, 2015). Moreover, the E/I balance in the SCN has shown to be modulated within a presumably "healthy" range under normal physiological conditions (Albus *et al.*, 2005; Choi *et al.*, 2008; Farajnia *et al.*, 2014).

2. E/I BALANCE IN THE SCN

In the experiments described in this thesis, we measured the E/I balance in SCN slices of mice under different circumstances, like changing light regimes or in aging. The E/I ratio was calculated by determining the GABAergic response of individual SCN neurons using calcium imaging. By applying this imaging technique, we were able to measure about 80 – 120 neurons per SCN, divided over 2 – 3 slices. This way, the different regions of the SCN – both anterior to posterior and ventral to dorsal – were represented in the measurements to ensure a proper distribution. We showed an increase in GABAergic excitation and E/I balance in the aged SCN (**chapter 4**) and after exposure to long photoperiod (**chapter 5**). We also demonstrated a decrease in inhibitory responses after exposure to skeleton long photoperiod (**chapter 2**).

The neurotransmitter GABA is mainly inhibitory to most adult neurons and because of its abundance it plays a critical role in setting the E/I balance. There are, however, parts of the mature brain, among which the SCN, with GABAergic depolarization and excitation in physiologically healthy states (Chung, 2012). In the SCN, GABA has the ability to act both inhibitory and excitatory and because most SCN neurons contain GABA, it is believed to be a major contributor to the E/I balance in the SCN (Wagner *et al.*, 1997; Albus *et al.*, 2005; Choi *et al.*, 2008; Irwin & Allen, 2009).

Previous studies have shown plasticity in the E/I balance in the SCN. For instance, there is a daily modulation of the E/I ratio in the SCN with more GABAergic excitation during the night compared to the day (Choi *et al.*, 2008; Irwin & Allen, 2009; Farajnia *et al.*, 2014). Also, the length of the day impacts the E/I balance, as the E/I ratio increases in SCN slices of mice adapted to a long day photoperiod, when compared to a short day photoperiod (Farajnia *et al.*, 2014; Myung *et al.*, 2015). Hence, the E/I balance fluctuates over the different seasons. These E/I changes seem functional, since plasticity in $GABA_A$ signaling was shown to be crucial for neuromodulation of the SCN network when adapting to different photoperiods (Rohr *et al.*, 2019). Several studies demonstrated that GABAergic excitation occurred mostly in the dorsal part of the SCN (Albus *et al.*, 2005; Choi *et al.*, 2008; Irwin & Allen, 2009). We therefore always tested for regional variances, but found no differences in the distribution of the different GABAergic responses along the dorsoventral axis. The lack of regional differences in GABAergic response types is comparable to a previous study examining the effect of photoperiod on the GABAergic responses in the mouse SCN (Farajnia *et al.*, 2014). The difference in spatial organization between our studies and the studies showing more excitation in the dorsal area of the SCN are difficult to explain, but it probably involves functional and anatomical differences in the brains of mice and rats (Morin *et al.*, 2006).

As the $GABA_A$ receptor is a GABA-gated chloride channel, the polarity of the GABAergic signal depends on the intracellular chloride concentration ($[Cl^-]_i$) and the relationship between the chloride equilibrium potential (E_{Cl}^-) and the membrane potential (V_m) (Kaila, 1994; Ben-Ari, 2002). Different classes of cation-chloride-cotransporters regulate $[Cl^-]_i$, of which at least two are expressed in the SCN; the chloride importer NKCC1 and exporter KCC2 (Belenky *et al.*, 2008). KCC2 extrudes Cl^- from the cell, thus when KCC2 is expressed and GABA binds to its receptor, the channel opens and because of the low $[Cl^-]_i$ there is an inward flow of Cl^- causing an inhibitory response. When NKCC1 expression dominates in neurons, the $[Cl^-]_i$ is relatively high, which results in an outward flow of Cl^- and subsequently an excitatory GABA response, after GABA binds to its receptor.

3. HOW DOES LIGHT INFLUENCE THE E/I BALANCE IN THE SCN?

As described above and in more detail in **chapter 1**, the time of the day and the length of the day influence the E/I balance in the SCN. In **chapter 5**, we confirmed that when mice were exposed to long photoperiods (i.e. 16 h of daylight), the E/I balance in SCN slices shifts towards more excitation and when exposed to short photoperiods (i.e. 8 h of daylight) it shifts towards more inhibition. In **chapter 5** we also demonstrated that the chloride cotransporter KCC2 – and thus the $[Cl^-]_i$ – plays a major role in the establishment of the E/I balance in the SCN. When KCC2 was blocked with the highly specific antagonist ML077, resulting in an increase in $[Cl^-]_i$ of the SCN cells, the polarity of the GABAergic response in many SCN neurons changed within minutes after application and the overall E/I balance got larger. This increase in E/I balance was evident and similar in all the experiments using ML077, indicating that KCC2 plays a role in regulating $[Cl^-]_i$, regardless of the baseline $[Cl^-]_i$ set by adaptation to long or short photoperiod. Interestingly, blocking KCC2 with ML077 in the SCN of mice adapted to short photoperiod resulted in an E/I balance that is very similar to the E/I ratio under long photoperiod (**chapter 5, figure 4A and 4E**). This showed that ML077

is a suitable tool to manipulate the E/I balance and can be useful for researching the functional mechanisms of E/I balance in seasonal encoding. More recent work focused on the role of both cotransporters NKCC1 and KCC2 in regulating $[Cl^-]_i$ in different parts of the SCN (Klett & Allen, 2017). NKCC1 contributed to $[Cl^-]_i$ regulation, however, KCC2 was shown to be the primary regulator. Interestingly, it seems to be differentially regulated between VIP and AVP (i.e. core and shell) expressing neurons, because blocking KCC2 had a larger effect on VIP neurons, when compared to AVP neurons (Klett & Allen, 2017). Additionally, the switch in GABAergic signaling after adaptation to long photoperiod corresponded with KCC2 downregulation in the core region of the SCN (Rohr *et al.*, 2019). Another study showed that NKCC1 protein expression was increased in the ventral SCN of hamsters exposed to a photoperiod of LD 14:10, when compared to the dorsal part (McNeill *et al.*, 2020). Concluding, the data from these studies indicate a role for both NKCC1 and KCC2 in the polarity of the GABAergic response in SCN neurons, though to a different extent, and thereby on the SCN network plasticity when challenged with environmental changes.

There are studies proposing that the variability in GABAergic responses in the SCN could support adaptation to environmental conditions and is therefore functional (Farajnia *et al.*, 2014; Myung *et al.*, 2015; Rohr *et al.*, 2019). The exact mechanism of how changes in E/I balance will contribute to phase distribution of SCN neurons is still under debate and has to be further investigated (McNeill *et al.*, 2020). A recent study on the expression level of NKCC1 in the SCN of hamsters showed increased protein levels after exposure to constant light, suggesting that light duration may influence the expression of NKCC1. However, when the hamsters were exposed to a long day of LD 14:10, the NKCC1 protein levels were not different from those of hamsters housed under constant darkness, suggesting that specific ratios of GABAergic excitation/inhibition are not required for seasonal entrainment (McNeill *et al.*, 2020). But, as described above, KCC2 might play a more prominent role in setting $[Cl^-]_i$ in SCN neurons than NKCC1 (Klett & Allen, 2017) and exposure to long photoperiod has resulted in KCC2 downregulation (Rohr *et al.*, 2019). Since entrainment to longer photoperiods decreases the magnitude of phase shifting effects of light (Pittendrigh *et al.*, 1984; Evans *et al.*, 2004; vanderLeest *et al.*, 2009), the increased E/I ratio in SCN neurons could also influence the phase shifting response of the SCN to light. However, in vivo injections of an NKCC1 inhibitor into the SCN decreased light induced phase delays, indicating that the excitatory effects of GABA in the SCN contribute to the phase delaying effect of light (McNeill *et al.*, 2018). These results seem to contradict our proposed consequence of increased E/I levels, considering that the behavioral phase shifts are smaller and the amount of excitatory GABA is higher in animals entrained to long photoperiods (vanderLeest *et al.*, 2009; Farajnia *et al.*, 2014). A possible explanation is that the acute use of cotransporter blockers, and the resulting acute change in chloride levels, is mechanistically different from adaptation to long photoperiod. In line with this, as demonstrated in **chapter 5**, application of the KCC2 blocker ML077 also show that, regardless of the baseline chloride levels, there is an additive, acute effect of the blocker. After entrainment to long photoperiod, cotransporter expressions and chloride levels in the SCN cells are changed, but this is a gradual change that takes weeks and is accompanied by a change in phase distribution. Studies based on experimental data, strengthened with modeling data, indicate that the chloride homeostasis and the polarity of the GABAergic signaling influence the coupling within the SCN

network and ultimately lead to phase adjustment (DeWoskin *et al.*, 2015; Myung *et al.*, 2015). It is likely that the decreased phase-shifting capacity, as seen after entrainment to long photoperiod, is the result of the increased phase dispersal in the SCN network. This, in turn, will result in a change in phase distribution, which has been shown to be the critical factor determining the phase shifting capacity in the SCN (vanderLeest *et al.*, 2009).

3.1. Brief light exposure influences the E/I balance in the SCN

In **chapter 2** we examined whether the reception of the full photoperiod was needed for photoperiodic encoding in the SCN at the level of network reorganization. As mentioned in the beginning of this chapter, seasonal encoding is a great example of cellular organization within the SCN network and its functional role. The SCN adjusts its ensemble electrical output to the length of the day of the changing photoperiod, caused by a change in distribution of phases of the individual SCN neurons (Mrugala *et al.*, 2000; VanderLeest *et al.*, 2007). Moreover, seasonal related network changes also induce changes in the E/I balance (Farajnia *et al.*, 2014) (**chapter 5**). In **chapter 2** we aimed to investigate which of these network changes occur when the circadian system is exposed to skeleton photoperiods, i.e. exposed to brief light pulses that mark the beginning and end of the day.

We showed that at different levels – ranging from molecular and cellular to behavioral – all characteristic changes when encoding for full long photoperiod, could be mimicked by just two light pulses of 30 minutes marking the beginning and end of the day. When mice were exposed to skeleton long photoperiod, the waveform of the ensemble electrical activity in SCN slices was broader than in slices from mice exposed to skeleton short photoperiod which is equivalent after entrainment to the full photoperiod. Similar increase in phase distribution was also shown by imaging single cell per2 clock gene expression in SCN slices of mice entrained to skeleton long photoperiod, compared to skeleton short photoperiod. Moreover, like entrainment to full long photoperiods, exposure to skeleton long photoperiod resulted in a compression of the behavioral activity pattern and a reduction in the magnitude of phase delays in response to a light stimulus, when compared to skeleton short photoperiod. Lastly, there was less GABAergic inhibition in SCN slices of mice adapted to skeleton long photoperiod compared to skeleton short photoperiod, although the overall E/I balance did not differ between the skeleton long and short photoperiod. Taken together, these results showed that brief light exposure at the beginning and end of the day are sufficient for photoperiodic encoding at the network level of the SCN.

The lack of a significant difference between the E/I ratios of skeleton long and short photoperiod, when compared to the full photoperiods, is based on a higher E/I balance in skeleton short photoperiod. Thus, the E/I ratio under skeleton short photoperiod is higher compared to a full short photoperiod. Remarkably, skeleton long photoperiod affected the SCN network to the same degree as full long photoperiod, but there are more differences between skeleton and full short photoperiod. The waveform of the ensemble electrical activity has a more compressed peak under full short photoperiod, when compared to skeleton short photoperiod. Also, the behavioral phase shift is less pronounced in animals entrained to skeleton short photoperiod compared to full

short photoperiod. The similarities between skeleton and full photoperiod when encoding long photoperiod and the differences when encoding for a short photoperiod suggest an additive effect of the full photoperiod. This is in line with recent evidence showing that specific components of light cycles have distinct effects on the circadian system (Tackenberg *et al.*, 2020). Previous work proposed that seasonal encoding depended on a subset of SCN neurons that respond to tonic photic cues, suggesting that seasonal adaptation would require exposure to the full photoperiod (Yan & Silver, 2008). This is in contrast to our work which clearly shows that characteristic cellular reorganization in the SCN, after adapting to long photoperiod, can be achieved by exposure to two brief light pulses. One explanation could be that the previous work was conducted in Syrian hamsters, whereas we worked with mice. Syrian hamsters are, in contrast to our mice, seasonal breeders that contain melatonin. Seasonal encoding by the SCN might be mechanistically different between these species.

The implications of the work from **chapter 2** are interesting, not only in terms of circadian adaptation mechanisms, but also from an ecological point of view. In the modern society, the use of electrical light at night increased immensely in the past decades causing disturbances in the activity patterns of nocturnal animals (Dominoni *et al.*, 2016; Sanders & Gaston, 2018). The skeleton photoperiods that we used actually resemble the natural lighting situation more closely compared to full photoperiods, at least for nocturnal animals. Many nocturnal animals sleep underground, thereby creating their own skeleton photoperiod. The electrical light at night, especially in wintertime, can be harmful to these animals considering the light pulses late at night can be interpreted as a sign for summer. This, in turn, can lead to maladaptive physiological behavior, like the production of offspring at the wrong time of year.

Chapter 2 underscores that even brief light exposure can have physiological effect and should be considered in light management programs aimed at protecting the clock function of nocturnal rodents in the natural environment.

4. AGING

The above mentioned fragmentation of light is also interesting in terms of optimizing light therapy to help the circadian rhythms in the elderly (Goudriaan *et al.*, 2021; Rubiño-Díaz *et al.*, 2021). In **chapter 3 and 4** several aspects of the circadian system – behavioral, molecular, and cellular – were investigated to further identify components that might be affected by aging and thus could be potential targets for such chronotherapeutic interventions. We showed that with aging, mice have a reduced ability to behaviorally adapt to short photoperiods, but there were no impairments in the PER2 rhythms after photoperiodic encoding (**chapter 3**). Additionally, the proportion of GABAergic excitatory responses and the subsequent E/I balance increased in SCN slices of aged mice (**chapter 4**).

Aging is an unavoidable and irreversible process that affects many aspects of physiology and behavior, including the central nervous system. Brain alterations due to aging are evident on various levels with, for instance, a decline in brain volume (Resnick *et al.*, 2003), loss of synaptic function (Jacobs *et al.*, 1997), and a decay in neurotransmitter function (Mora *et al.*, 2007). As detailed

reported in **chapter 1**, aging also affects the circadian system on multiple levels. On the behavioral level, there are age-related disruptions in locomotor activity that lead to more fragmented sleep-wake patterns and an overall reduction in behavioral activity (Valentinuzzi *et al.*, 1997; Dijk & Duffy, 1999; Farajnia *et al.*, 2012). Aging is also associated with reduced synchronization and diminished amplitude of electrical activity rhythms in the SCN neuronal network (Nakamura *et al.*, 2011; Farajnia *et al.*, 2012). Furthermore, at the cellular level, the physiology of the SCN neuron itself is impacted by aging (Aujard *et al.*, 2001; Farajnia *et al.*, 2012). Long term disruption of circadian rhythmicity can be detrimental and is associated to age-related diseases (Kondratova & Kondratov, 2012; Steponenaite *et al.*, 2018). Thus, understanding the mechanisms of aging-related clock disorders will allow targeting for repair of the circadian system.

4.1. Aging affects the E/I balance in the SCN

As mentioned earlier this chapter, alterations in the E/I balance occur at the cellular level, with neurons being inhibitory or excitatory, but likely also contribute to network changes. In **chapter 4** we aimed to investigate whether there is an aging-effect on the GABAergic E/I balance in the SCN, that could contribute to the aging-related network alterations. By measuring GABA stimulated changes in Ca^{2+} transients, we observed significantly more excitatory responses in SCN slices from old mice, compared to young mice. In addition, in the posterior SCN from old mice, we showed a significant decrease in inhibitory responses. Also, the baseline Ca^{2+} levels in the old SCN neurons was higher compared to SCN neurons from young mice. These results demonstrated that aging affects the polarity response, and thus the E/I balance, in the SCN and indicated that the Ca^{2+} homeostasis is altered in the aged SCN network. Whether the increased E/I balance has functional relevance to the aging clock, or is a sign of loss of function remains unclear. Cortical excitability has been associated with age-related cognitive decline. A reduction in circadian rhythms of cortical excitability was found in aged human volunteers during sleep deprivation, which was likely the consequence of a diminished impact of sleep homeostasis and might underlie the reduction in cognitive flexibility in aging (Gaggioni *et al.*, 2019). Alterations in E/I balance also occur in age-related diseases like Alzheimer's Disease (AD) and may be a primary mechanism contributing to seizure activity and cognitive decline in AD patients (Rissman & Mobley, 2011; Bi *et al.*, 2020). Recently, evidence from human post-mortem parietal cortex samples of individuals with AD demonstrated elevations in the synaptic E/I ratios that contributed to cortical hyper-excitability and cognitive impairment in AD patients (Lauterborn *et al.*, 2021). Besides the cognitive decline in AD patients, there are numerous studies that have shown an age-related shift in the E/I balance with heightened hippocampal and prefrontal cortical activity which contributes to memory-impairment (Legon *et al.*, 2016; Tran *et al.*, 2019; Koh *et al.*, 2020). Aged rats without cognitive impairment most likely have a compensational mechanism as they display increased inhibitory postsynaptic currents in recordings from dentate gyrus cells and a larger tonic inhibitory current in the pyramidal neurons, which is lacking in rats with memory impairment (Tran *et al.*, 2018; Tran *et al.*, 2019; Koh *et al.*, 2020). This emphasizes the importance of an adequate balance between inhibition and excitation, also for healthy aging.

Within the SCN, several lines of evidence suggest a possible role for the E/I balance in synchronization mechanisms (Farajnia *et al.*, 2014; Myung *et al.*, 2015; Rohr *et al.*, 2019). Others presented results that GABA plays a role in (de)synchronization, but without distinguishing between the inhibitory or excitatory action of GABA (Liu & Reppert, 2000; Albus *et al.*, 2005; Aton *et al.*, 2006; Evans *et al.*, 2013; Freeman *et al.*, 2013). We showed in **chapter 2, 4 , and 5** alterations in the distribution of GABAergic responses, with more excitation and/or less inhibition, in SCN slices in which the phases of the individual neurons were more distributed. In aging (**chapter 4**), under long photoperiod (**chapter 5**), or after exposure to skeleton long photoperiod (**chapter 2**) the degree of synchronization is lower compared to the young SCN or the SCN of mice exposed to short (skeleton) photoperiod (**chapter 2**) (VanderLeest *et al.*, 2007; Farajnia *et al.*, 2012). Although the mechanisms that regulate neuronal phase distribution in the SCN are still unknown, the results from this thesis consistently indicate that changing the amount of GABAergic inhibition or excitation in the SCN contribute to the network alterations. Future research with in vivo measurements of E/I balance in the SCN during entrainment to different photoperiods would help to elucidate the role of E/I balance in seasonal encoding and network reorganization. We consider the seasonal changes in the E/I balance as a functional and physiological process, supporting clock function. The question remains whether this is also the case with aging and if there is still plasticity in the E/I ratio in the aged SCN.

4.2. Aging affects calcium levels in the SCN

Ca^{2+} is an important intracellular component involved in phase adjustment and network stability. Besides a shifted E/I balance in the aged SCN, the data from **chapter 4** suggest that aging causes an altered Ca^{2+} homeostasis as the baseline Ca^{2+} levels are higher in SCN neurons from aged mice. These increased baseline levels of Ca^{2+} could impact cellular phase adjustments, as Ca^{2+} is implicated in rhythm generation and light-induced phase shifts by activating clock gene expression (Kim *et al.*, 2005; Golombek & Rosenstein, 2010). With aging, phase shifting capacity and network stability could be reduced due to the increase in $[Ca^{2+}]_i$. Research has shown a circadian rhythmicity in $[Ca^{2+}]_i$ with higher levels during the day, compared to the night (Colwell, 2000; Ikeda *et al.*, 2003) and one study demonstrated that this rhythm is reversed in old mice (Farajnia *et al.*, 2015). The latter, however, did not find differences in Ca^{2+} levels during daytime, when compared with young controls, which is in contrast to our results on baseline Ca^{2+} levels. Since Ca^{2+} is a key cell signaling molecule and an important link between the molecular clock and electrophysiological properties of the SCN neurons, Ca^{2+} could be an interesting target for intervention aimed at strengthen the clock in aging.

4.3. Photoperiodic encoding is affected in aging

In **chapter 2 and 5**, we showed that photoperiodic entrainment requires plasticity of the SCN network and in **chapter 4** we demonstrated that aging causes changes in the E/I balance and Ca^{2+} levels in the SCN cells. **Chapter 3** aimed to study if, and how, seasonal encoding is affected by aging by investigating the plasticity of both behavior and clock gene expression rhythms. We showed that aging does not affect the expression pattern of the clock gene PER2 in an equinoctial light regime, while the behavioral rhythm strength in the aged mice declined. Exposure to long or short

photoperiods requires plasticity of the SCN network (VanderLeest *et al.*, 2007; Meijer *et al.*, 2010; Porcu *et al.*, 2018) which is displayed, for instance, by changing the phase distribution of single cell PER2 expression rhythms (Buijink *et al.*, 2016). Surprisingly, the PER2::LUC expression rhythms of the SCN of old mice showed similar levels of phase distribution as the SCN of young mice, with a wider phase distribution after adaptation to long photoperiod as compared to short photoperiods. In contrast to the intact molecular clock of old mice, behavioral rhythms were less adjusted to the changing photoperiod. This demonstrates that most of the plasticity of the molecular clock remains intact with aging, and deficits in photoperiodic adaptation, like the increased E/I balance that we showed in **chapter 4,** arise downstream from the molecular clock.

Studies on the effect of aging on other components of the core molecular clock also indicate that the molecular clock in SCN neurons remains largely functional, although more research is required to fully confirm this (Banks *et al.*, 2016; Buijink & Michel, 2020). If so, several possibilities exist that could explain the age-related deficits of the circadian clock. Specifically, aging causes a dampened amplitude of the SCN output signal that is expected to be the result of reduced synchronization within the network (Nakamura *et al.*, 2011; Farajnia *et al.*, 2012). When the SCN amplitude is diminished, electrical activity has less influence on PER2 expression rhythms (Noguchi *et al.*, 2017) and circadian rhythms in electrical activity, Ca^{2+} and the molecular clock can become dissociated (Enoki *et al.*, 2017a; Enoki *et al.*, 2017b). Aging affects the rhythm and baseline levels (**chapter 4**) of intracellular calcium in SCN neurons. This disturbed calcium homeostasis could account for a weakened link between the molecular clock and the SCN network. Moreover, the E/I balance is shifted in aging (**chapter 4**) suggesting that the E/I ratio is downstream from the molecular clock and influenced by other mechanisms.

5. CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this thesis, I was able to demonstrate that the SCN network is plastic and under different conditions – like exposure to different light regimes or under the influence of aging – it can reorganize the phase relationships between the individual neurons. Even though characteristics of individual SCN neurons can also change due to environmental influences, it is the network that is of critical importance for proper adaptation. The communication and synchronization between the SCN neurons is key in entrainment and loss of intercellular communication can cause decrement of the network functionality. Therefore, a better understanding of the functioning of the SCN network, and especially the (repulsive) coupling among the SCN neurons, could provide insights in how to deal with the circadian disturbances and challenges in our modern lifestyle or aging. The work in this thesis suggests that the GABAergic E/I balance in the SCN plays a role in network changes and could be a possible target for interference when circadian rhythms are disturbed. There are several pharmacological tools that can manipulate E/I balance, but their application as therapeutics might be challenging due to difficult pharmacokinetics (Gagnon *et al.*, 2013). There are, however, novel therapies that aim to normalize E/I ratio (Ghatak *et al.*, 2021) which could also be interesting for circadian research. These are promising targets to strengthen the circadian clock in the case of disturbances due to environmental light or due to aging.

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