Network properties of the circadian clock: an integration of computational methods and empirical data

Beurden, A.W. van

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General discussion

The central circadian clock of mammals consists of a network of coupled oscillators located in the suprachiasmatic nucleus (SCN). Circadian rhythms in electrical activity function as output of the SCN and are generated by the complex interplay between SCN neurons. The research in this thesis aimed at identifying network properties of the circadian clock by investigating the four functional components of the SCN: (1) light input to the SCN, (2) neural network synchronization within the SCN, (3) output from the SCN to brain and body, and (4) feedback from these processes back to the SCN. We used empirical data together with computational methods to obtain new insights in the network properties of the circadian clock. We furthermore identified similarities and differences in the SCN network between nocturnal and diurnal species. As there are many diseases affecting clock function, understanding the SCN network is a first step towards maintaining health. In this chapter I will give some recommendations on how to strengthen clock function based on the results from the previous chapters.

1. Light input to the SCN

1.1 Light-excited neurons in the SCN initiate entrainment to shifted light cycles

Phase shifts are frequently occurring in modern day society. Not only the most evident sources of phase shifts, such as engaging in rotating shift work or traveling to a different time zone, cause misalignment of the circadian clock with the external light-dark cycle. Also less evident sources, like exposure to artificial light at night and social jetlag, which is characterized by a difference in the sleep/wake cycle between free days and workdays, cause misalignment of the clock (Caliandro *et al*., 2021; Vetter, 2020). Repeated phase shifts of the internal clock have profound negative health consequences, such as metabolic disruptions, obesity, cardiovascular disease and cancer (McFadden *et al.,* 2014; Reinke & Asher, 2019). Given the frequent occurrence of phase shifts, understanding the resetting process is important. Although the resetting process after a phase shift has been studied before (Albus *et al*., 2005; Rohling *et al*., 2011), this was never investigated at the single-cell level.

In **Chapter 2** we investigated the response of the SCN to a 6-h phase delay of the light-dark cycle at the single-cell level in mice. We found a broadened phase distribution in PER2 expression after the delay, which is in agreement with previous studies investigating the resetting process at the population level (Davidson *et al*., 2009; Nagano *et al*., 2003; Nakamura *et al*., 2005). In more detail, we found that a subpopulation consisting of approximately 30% of the neurons in the SCN undergo a rapid phase shift, while the other neurons shift more slowly. This can be seen as a bimodal distribution in PER2 expression at the population level. We furthermore found that the neurons that shift rapidly after a delay spatially overlap with neurons that respond with excitation to stimulation of the optic nerve. These results suggests that light-excited neurons in the SCN initiate entrainment to a shifted light-dark

cycle. This role for the light-excited neurons in the SCN in photoentrainment has never been made explicit before, but fits nicely with the current models on photoentrainment, in which depolarization leads to phase shifts (Colwell, 2001; Colwell, 2011).

Note that this extension of the current models on photoentrainment might only apply for nocturnal animals. In mice the vast majority of responses in the SCN upon optic nerve stimulation are excitatory, whereas in diurnal *Rhabdomys pumilio* there are approximately an equal amount of excitations and inhibitions (Schoonderwoerd *et al*., 2022a). The role of inhibitions in photic entrainment is unknown, and the low percentage of inhibitory responses in mice in our phase delay study, did not allow to determine their functional role. However, the increased proportion of light-inhibited cells in *Rhabdomys* could reflect a difference in the photoentrainment mechanisms between nocturnal and diurnal animals.

To strengthen clock function, first of all the exposure to phase shifts of the light-dark cycle should be minimized, as every phase shift causes internal desynchronization in the SCN and in the rest of the body. Some types of phase shifts, like traveling to a different time zone, are unavoidable, but other types of phase shifts, like exposure to artificial light at night, can be avoided. Although the spectrum and intensity of artificial light are not the same as of daylight (Hut *et al*., 2000), artificial light can induce phase shifts. Exposure to artificial light at night should therefore be prevented as much as possible. The general advice to use a blue-light filter in the evening may not be sufficient to prevent phase shifts, as a recent study shows that the SCN is sensitive to a broader spectrum of wavelengths than the spectrum of blue light only (Schoonderwoerd *et al*., 2022b).

If the phase shift is unavoidable, there are still a couple of things to take into account to minimize the negative health effects and to strengthen the clock. I would recommend to shift all daily activities directly to the new light dark regime, in order to fulfill the phase shift as quickly as possible. By not shifting all daily activities directly, the period of misalignment is enlarged, possibly worsening clock function. So, there should be exposure to the bright daylight and food intake should take place at regular times. One exception to this advice is for rotating shift work, because it is not possible to fully shift all rhythms back and fort every couple of days.

2. Neuronal network synchronization within the SCN

2.1 Quantifying coupling strength between neurons in the SCN

The network structure of the SCN is organized in such a way that there is a fine balance between flexibility on the one hand and robustness on the other hand (Zheng *et al*., 2022). Flexibility of the clock is required in order to adjust to changes in the environment, while robustness is necessary for the clock to be resistant to small external perturbations. Encoding of seasonality is one of the processes that requires

plasticity of the clock (Meijer *et al*., 2010). Seasonality is encoded at the network level of the SCN (Schaap *et al*., 2003; Hazlerigg *et al*., 2005; Vanderleest *et al*., 2007). The study of Buijink *et al*. (2016) found evidence that there is weakened intercellular coupling in long photoperiod, compared to short photoperiod.

In **Chapter 3** we used a Kuramoto model to estimate the coupling strength within and between neuronal subpopulations of the SCN. We used PER2 gene expression data from young and aged mice entrained to short and long photoperiod as input for the model. Although the empirical data did not allow to estimate the exact coupling strengths, we could estimate the coupling strengths under the different conditions relative to each other. It appeared that young and aged mice both show weaker coupling in long photoperiod than in short photoperiod. We furthermore found that in short photoperiod young mice are able to increase their neuronal coupling strength over a larger range than aged mice. In aged mice the capacity to reach stronger coupling is diminished. The inability of aged mice to reach stronger coupling likely contributes to the behavioral inability of aged mice to adapt to short photoperiod (Buijink *et al*., 2020).

It was previously reported that the effects of aging were mainly visible in the SCN electrical activity rhythm (Leise *et al*., 2013; Nakamura *et al*., 2011; Farajnia *et al*., 2012) and downstream of the SCN, but not in the molecular clock (Buijink & Michel, 2021). In **chapter 3** we also found no differences in the phase coherence of PER2 expression between young and aged mice, which is in agreement with these studies. However, we did find a difference in coupling strengths between young and aged mice, based on PER2 gene expression, by use of the 2-community Kuramoto model. This shows the ability of the Kuramoto model to determine network properties of the SCN that are not directly measurable from the empirical data and underlines the importance of the use of models in general.

Although the phase coherence of PER2 expression in the SCN is higher in short photoperiod than in long photoperiod (Buijink *et al*., 2016), this does not automatically mean that the rhythm output is stronger and behavioral adaptation is easier. Based on the results of the Kuramoto model, it seems like it does not matter for young mice whether they are subjected to short or long photoperiods, in order to maintain strong circadian rhythms. For aged mice, on the other hand, it is unfavorable to subjected them to short photoperiods, in order to maintain strong circadian rhythms. Here we used a light-dark regime of 8 hours of light and 16 hours of darkness for short photoperiod and vice versa for long photoperiod. Adaptation to more extreme photoperiods can even be difficult for young mice, and is related to negative health consequences (Tackenberg & McMahon, 2018). For instance, in constant light, which can be considered the most extreme long photoperiod, mice are prone to metabolic dysfunction and they become arrhythmic in their behavior (Ohta

et al., 2005; Wyse *et al*., 2014). Therefore, I would recommend to stay as close as possible to an equinox photoperiod to strengthen circadian rhythms.

2.2 Drugs to enhance circadian rhythms

There are multiple ways to strengthen circadian rhythms. I have already given some recommendations in this chapter, such as minimizing exposure to artificial light at night. Although these recommendations can be beneficial to strengthen circadian rhythmicity, they might not be sufficient for some patient groups and for the elderly, in which circadian rhythms are more heavily disturbed (Buijink & Michel, 2021). This askes for a different strategy to enhance circadian rhythms. Drugs that are designed to modify circadian rhythm properties could be a solution. High throughput chemical screenings have already pointed towards some synthetic small molecules that have the potential to enhance circadian rhythms (Chen *et al*., 2012; Doruk *et al*., 2020; He *et al*., 2016; Li *et al*., 2022). Clock Enhancing Molecule 3 (CEM3) is a small molecule that has shown to increase the SCN rhythm amplitude at the tissue level (Chen *et al*., 2012).

Both an increase in the amplitude of the individual neurons as well as an increase in synchrony among the neurons can result in an enhanced rhythm amplitude at the tissue level. To investigate the mode of action of CEM3, we measured the response of the SCN to application of CEM3 at the single-cell level in **Chapter 4**. We compared the amplitude and phase coherence of PER2 gene expression rhythms before and after application of CEM3. We found that CEM3 acts by enhancing the amplitude of the individual neurons in the SCN. CEM3 did not alter the period length or the phase coherence among the neurons.

The effect of CEM3 was largest in the first cycle after application. We therefore performed additional experiments in which we applied CEM3 at three consecutive cycles, to mimic taking a medicine daily. The second application appeared effective to keep the rhythm amplitude enhanced, but the third dose appeared ineffective. The third dose was most likely ineffective due to receptor desensitization. It needs to be tested whether the third dose of CEM3 would also be ineffective when applied in vivo, in view of the continuous refreshment of cerebrospinal fluid. Other recently discovered small molecules which enhance the amplitude of circadian rhythms, like CLK8 and ISX-9 (Doruk *et al*., 2020; Li *et al*., 2022), have not been tested with repeated application. So, unfortunately we cannot compare if the same would happen with these compounds.

One factor that we did not investigate for the application of CEM3, but which is receiving more and more attention in testing of drugs, is the timing of application of the drug. In most recent studies in which time of application is explicitly investigated, there are clear time-of-day dependent variations in drug efficacy or toxicity (Cederroth *et al*., 2019; Ruben *et al*., 2019; Ruan *et al*., 2021). We have applied CEM3

at the exact same phase of the 24-h cycle in all experiments, and for this specific timepoint we found amplitude enhancing effects. It is possible that application of CEM3 at a different phase of the 24-h cycle would either be more or less effective.

It is probably quite obvious how CEM3 could strengthen clock function. CEM3 could be used as a medicine to enhance the amplitude of circadian rhythms in the elderly as well as in specific patient groups. It is beneficial that CEM3 does not alter period length or interferes with neuronal synchronization, because that could complicate other functions of the clock, like entrainment to the light-dark cycle or daylength encoding (Schaap *et al*., 2003). Of course, there is still a long way to go before CEM3 could possibly be approved as medicine by the EMA. But the mode of action of CEM3 provides a good example, showing the potential of small molecules to restore low amplitude circadian rhythms. CEM3 may be used as a starting point for the development of future therapeutics.

3. Output from the SCN and feedback to the SCN

3.1 Interplay between the SCN and physical activity

In our modern day society many people are sitting for large parts of the day and they are not having a very active lifestyle in general. This is a big problem, as a sedentary lifestyle can weaken circadian rhythmicity (de Souza Teixeira *et al*., 2020). As previously explained, the timing of physical activity is not only an output of the circadian clock, but in turn affects the clock. Whereas properly timed exercise can enhance the amplitude of circadian rhythms, lack of exercise, or exercise at timepoints when the animal is supposed to rest can weaken the amplitude of circadian rhythms (Caputo *et al*., 2024; Van Oosterhout *et al*., 2012).

In **Chapter 5** the method of convergent cross mapping was used to quantify the bidirectional influence between the brain and behavior. We applied this method to simultaneously recorded time series, containing the SCN impulse frequency and the animal's behavioral activity, from nocturnal mice and diurnal arvicanthis. In mice, we found that the influence from the SCN to behavior is larger than the influence of behavior on the SCN. In arvicanthis, on the other hand, we found that the influence is similar in both directions. Moreover, the bidirectional influence between the SCN and behavior appeared to be rhythmic in mice, as well as in arvicanthis. The influence in both directions is largest when the animal is active and smallest when the animal is in rest.

By manipulation of the amount of physical activity in mice, we observed that increased levels of physical activity can strengthen the influence of behavior on the SCN. The effects of exercise are acute and temporary, i.e. the influence of behavior on the SCN is only increased during the active period of the animal, but not during the rest period. In the rest period, the influence from behavior on the SCN does not differ from mice without increased levels of physical activity. Increasing the tightness of

the loop between the SCN and behavior could represent an additional mechanism to strengthen clock function.

In arvicanthis the influence from behavior on the SCN is larger than in mice. As increased physical activity strengthens the influence of physical activity on the SCN, one intuitive explanation would be the fact that arvicanthis are naturally more active than mice (Hubbard *et al*., 2015). However, the difference cannot be due to higher levels of physical activity in arvicanthis compared to mice, because the influence of physical activity on the SCN in arvicanthis in the rest phase is higher than in mice in the active phase. Therefore, it is more likely that there is a fundamental difference in the way the pacemaker is influenced by physical activity between diurnal and nocturnal animals.

Chapter 6 explores the potential differences in the SCN network between nocturnal and diurnal animals, based on the feedback of physical activity. We used a Poincaré model to simulate feedback from physical activity to the light-entrained SCN. From the model we could infer that the network topology for which the input from light and physical activity are optimally integrated are different for nocturnal and diurnal animals. In nocturnal animals the input from light and physical activity should act on the same subpopulation of neurons in the SCN, while in diurnal animals the input from light and physical activity should act on different neuronal subpopulations in the SCN.

Light input reaches the SCN via the retinohypothalamic tract (Morin & Allen, 2006). The photic input pathway to the SCN is well studied, and it is known that the afferent fibers project mainly to the ventrolateral part of the SCN (Challet & Pévet, 2003). The major nonphotic input pathways to the SCN consist of afferent fibers from the intergeniculate leaflet and the raphe nucleus (Morin, 2013). Less is known about which parts of the SCN are innervated by the nonphotic input pathways. The nonphotic input pathways to the SCN should be studied in more detail in future research, in order to investigate whether the hypothesis arising from the Poincaré model is true.

The major differences between nocturnality and diurnality are generally contributed to a sign reversal downstream of the SCN (Mrosovsky, 2003; Smale *et al*., 2003). It is however likely that the differences between nocturnality and diurnality are more complex than a simple sign switch. Differences in the input signals to the SCN, the SCN network and feedback into the SCN are often neglected. In **chapter 5** and **6** we found evidence for fundamental differences in the way that feedback from physical activity affects the circadian clock. While there certainly are differences downstream of the SCN contributing to diurnality, there should be more attention to additional differences between nocturnality and diurnality in all four functional components of the SCN. As most circadian research has been performed in nocturnal animals, these results may sometimes not be directly translatable to diurnal animals.

4. Concluding remarks

4.1 A proper functioning and healthy clock

Nowadays it has been widely accepted that a proper functioning and well-entrained clock promotes health, while disruptions of the circadian clock are linked to increased risk of several diseases (Kramer *et al*., 2022). Diseases such as diabetes (Stenvers *et al*., 2019), obesity (Bruzas & Allison, 2019), psychiatric disorders (Cosgrave *et al*., 2018) and immune dysfunction (Xiang *et al*., 2021) are all linked to disrupted clock function. It is not just the neuronal network synchronization within the SCN, which determines whether the clock is functioning properly. Also the three other functional components of the SCN (light input, output signals and feedback) are important for a healthy clock. This also means that a manipulation of one of the four functional components of the SCN can already contribute to the strengthening of circadian rhythms. Importantly, when manipulating the clock in order to strengthen clock function, the differences between the nocturnal and diurnal SCN must also be taken into account.

In the view of healthy aging, the focus of treating diseases should be shifted towards the prevention of these diseases. This seems especially interesting to explore for diseases which are related to disrupted clock function. In the case of disrupted clock function a positive feedback loop can arise, which amplifies the negative health effects. For instance, for a person with obesity it can be more difficult to get enough exercise. Subsequently, the lower level of physical activity can dampen the amplitude of the already disrupted circadian rhythms, which in turn increases the risk of several other diseases. It is therefore important to keep the circadian clock healthy to prevent pathologies. Currently, medicines to strengthen clock function are still in the early stages of development, but with the correct lifestyle choices it is possible to help strengthening circadian rhythms. By minimizing phase shifts of the clock, being sufficiently active during the proper time of day, and minimizing the use of artificial light at night the likelihood of a healthy circadian system increases. We need to learn to respect our internal clock.

4.2 Computational methods are essential in circadian research

Throughout this thesis different computational methods and mathematical models are used in combination with empirical data to provide insights that are not always directly visible from the data. The Kuramoto model which we used in **Chapter 2** showed for instance that SCN neurons of old mice have a reduced capacity to reach strong coupling strengths. Models can furthermore be used to generate hypotheses, which can be tested with empirical experiments. In this way it can be decided which experiments are worthwhile, in order to perform less animal experiments. Like in **Chapter 6**, where we used a Poincaré model to predict the potential differences in the SCN network between nocturnal and diurnal animals. Lastly, computational

methods can also be used to quantify the interaction strength between neuronal subpopulations or even between different levels of hierarchical organization, for which the interaction strength cannot be directly measured. With the method of convergent cross mapping we could for instance bridge the scale between the brain and behavior in **Chapter 5**. These examples demonstrate the additive value of computational methods in circadian research to reach scientific progress.

4.3 To conclude

In my view the SCN network is very fascinating. The small size of the SCN together with its oscillatory behavior, make it a very interesting brain area to investigate. On the one hand, the SCN network is very complex. The plasticity of the network makes it impossible to capture the entire functional and structural network structure in the span of a day, year, or lifetime. On the other hand is it very easy to simplify the SCN network. A simple model of two coupled oscillators can already capture many properties of the circadian clock (Pittendrigh *et al*., 1958). The right balance between complexity and simplicity needs to be found in the ongoing search for network properties of the SCN. Our efforts have contributed to a more detailed understanding of the network properties of the SCN. We have furthermore highlighted that there are several small network differences contributing to a diurnal phenotype. As evolution to diurnality occurred independently in different lineages (Roll *et al*., 2006; Smale *et al*., 2008), we cannot assume that the SCN of all diurnal species function exactly the same. It would therefore be a great next challenge to learn more about the network properties contributing to diurnality in a wider range of diurnal species.

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