The circadian system throughout the seasons of life

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Chapter 9

General discussion

1. General discussion

The studies described in this thesis assessed the mammalian clock on multiple levels, from molecules to behavior, and under different circumstances, namely varying daylength and aging. It is shown that 1) the properties of the circadian rhythms vary significantly across the different levels, and 2) that within a single organism, the response to changing circumstances varies across the different parts of the clock. For example, neurons in the anterior part of the SCN change their relative phase in rhythmicity to changes in daylength, while there is no noticeable adaptation in the posterior part. Moreover, while the adjustment of the molecular clock to changes in daylength is similar in young and old mice, aged mice do show deficits in adapting their behavioral to daylength. In addition, while rhythmicity in metabolite levels in blood plasma does not seem to change as a result of aging, the rhythmicity of metabolites in the liver markedly decreases with age. This illustrates the complexity of the study of circadian rhythmicity across different organizational levels and lifetimes, and highlights the importance of studying multiple parameters of the circadian system simultaneously. Moreover, these findings have consequences for the development of approaches to improve an individual's circadian rhythm. Integrating various strategies to strengthen the circadian system on multiple levels will likely maximize the health benefit from chronotherapy.

1.1 Adaptability of the molecular clock in the SCN

The neuronal network of the suprachiasmatic nucleus (SCN) needs to be robust for accurate and reliable timekeeping, as well as flexible and adaptative to respond to changes in the environment. In **chapter 3-5** we studied the plasticity of SCN neurons and their network. By using bioluminescence imaging of PERIOD2::LUCIFERASE (PER2::LUC) expression as a readout for the molecular clock, we assessed the response of the molecular clock in SCN neurons to changes in the light-dark cycle. The recording of PER2::LUC expression in individual neurons for multiple days enabled us to assess the impact of exposure to long and short photoperiods on the molecular clock at the single-cell and population level. Furthermore, we applied a novel analysis technique to identify functional clusters in the SCN neuronal network, based on the time-series data from single cells (**chapter 3**). The resulting clusters have a high internal positive correlation in temporal dynamics, while between the clusters, rhythm characteristics displayed a negative correlation, meaning that they behave in an opposite manner. The application of this method to our recordings of PER2::LUC in slices of the SCN typically resulted in two clusters of neurons of approximately the same size. Interestingly, these clusters also exhibited a very similar spatial pattern, which could mean that these populations of cells represent a functional group based on their expression of neurotransmitters and/or receptors. In **chapter 4 & 5** we examine the effects of photoperiod on rhythm characteristics in different areas of the

SCN, namely the anterior and posterior SCN, and of the two clusters that resulted from the method described in **chapter 3**.

There is a long history of searching for functional areas within the SCN that would explain its versatile functions in regulating temporal processes throughout the brain and body. More than 45 years ago, a model was developed that could explain several observed characteristics of circadian rhythms, such as adaptation to photoperiod, bimodal activity, after-effects of the light-dark cycle in constant darkness and splitting, which is the appearance of two circadian components with independent period lengths (Pittendrich and Daan, 1976). This model was based on two independent, but coupled morning (M) and evening (E) oscillators. Although the search for the M and E oscillators has to date not provided a clear answer with regards to its location or even its existence, it has provided a host of new insights in the function of the circadian system (Helfrich-Förster, 2009). For instance, it has led to the identification of spatial differentiation in electrical activity and clock gene expression in response to photoperiod.

In **chapter 4** we showed a spatial difference in response to long and short photoperiod: the anterior SCN responds to long photoperiod with a wider phase distribution in Per2 expression rhythms, compared to short photoperiod, while there is no difference in phase distribution between the two photoperiods in the posterior SCN. When we further investigated the rhythm characteristics within the identified clusters from **chapter 3**, we found that the clusters differed in average peak time. Moreover, the dorso-lateral region in the anterior SCN displayed a higher period variation in mice exposed to long photoperiod. This period variability could be a means to establish the wider phase distribution observed in electrical activity and in the expression rhythms of several clock genes in long photoperiod. This is informative of the mechanism responsible for the phase dispersal: studies with dispersed neurons in vitro have shown that the lack of neuronal connections results in an increased period length instability, which suggests that a reduction in coupling could be responsible for phase dispersal in long photoperiod (Welsh *et al.*, 1995; Herzog *et al.*, 2004).

Our study corroborates previous findings that show an anterior-posterior difference in response to photoperiod. For instance, in Siberian hamsters, gene expression of the clock-related genes *per2*, *rev-erb*α and *dbp* clearly displayed distinct expression patterns in response to long vs short photoperiod in the anterior vs. posterior SCN (Hazlerigg *et al.*, 2005). In the murine SCN, studies using *in vitro* bioluminescence recordings of single-cell *per1* expression found that after exposure to long photoperiod, only in the anterior SCN gene-expression peaks were clearly divided into two populations, which were termed morning and evening cells by the authors (Inagaki *et al.*, 2007). A similar study of the mouse SCN, using different photoperiods to study the expression pattern of PER2 protein, found

the spatial distribution of phases resembling the "core" and "shell" region in the anterior SCN, previously identified in expression of the neuropeptides AVP and VIP (differences in anterior vs. posterior were shown in an earlier study without applying different photoperiods (Evans *et al.*, 2011; 2013). Surprisingly, the dorsal-lateral spatial distribution of the peak phase in PER2 protein expression by Evans et al. (2013) does not resemble the medial-lateral pattern we found in **chapter 3-5** for the same protein, while these studies used a very similar method and animal model. However, it is possible that the slightly different background of the mice (C57Bl/6J vs. C57Bl/6H), or a different cutting technique is responsible for this difference. Interestingly, for the core clock gene *Bmal1*, regions with a similar phase and period length were found in a lateral-to-medial distribution in the anterior SCN, gradually changing to a dorsal-to-ventral distribution in the central and posterior SCN (Myung *et al.*, 2012). In this case, the distribution of rhythm characteristics of *Bmal1* and *Per2* in the anterior SCN does resemble the pattern we observed for PER2, while the distribution in the posterior SCN is entirely different (ventral-dorsal for *Bmal1* and lateral-medial distribution for PER2).

The spatial distribution of the clusters based on PER2 expression, identified in **chapter 3** revealed a clear pattern that was very similar in all samples from the same regions of the SCN. Interestingly, this pattern does not match the patterns previously found for neuropeptide expression (Moore *et al.*, 2002; Wen *et al.*, 2020), nor electrical activity (Brown & Piggins, 2009). Moreover, it does not resemble the spatial distribution of clusters of cells with similar rhythm characteristics (e.g., peak time and period length) of other clock genes, like Per1 and BMAL1 ((Inagaki *et al.*, 2007; Myung *et al.*, 2012). Taken together it seems that the spatiotemporal dynamics of clock gene expression is considerably more complex than may be expected. The variety in spatial distribution of gene expression of e.g. clock genes, neurotransmitters and their receptors leads to multiple regions with unique expression patterns (Wen *et al.*, 2020). It is conceivable that the resulting spatial organization reflects functional areas, with specialized roles in the integration, representation and output of timing signals.

1.2 Circadian rhythms of metabolism and metabolites

The output of the SCN transmits timing information to peripheral tissue in multiple ways, mainly via other brain areas that in turn release hormones and other signaling molecules, or directly innervate peripheral tissues. The outputs from the SCN influence a wide variety of timed processes; from behavior, such as feeding and activity, to physiological processes, such as blood pressure and liver function. Circadian rhythms have primarily evolved to optimize energy metabolism and related processes. The correct timing of metabolic processes is essential to maintain a healthy organism. For example, the energy producing Krebs cycle also produces reactive oxygen species (ROS), that can damage other molecules in the cell, such as DNA. Therefore, it is important to activate DNA repair systems at the

same time as the activity of energy production (Cucchi *et al.*, 2021). Since metabolites are a reflection of cellular metabolic processes, assessing their dynamics over the day can give insight into 24-hour rhythms in these processes. The circadian system in the periphery functions in a highly tissue-specific manner. The phase of the molecular clock, the processes that are regulated, as well as the response to external timing signals varies widely with tissue type (and even with cell type; (Schibler & Sassone-Corsi, 2002; Harder & Oster, 2020; Kinouchi *et al.*, 2021). Moreover, there is mounting evidence that peripheral tissues can exchange timing signals, independent of the SCN (Zhang *et al.*, 2020). The diversity and interdependence of peripheral clocks mean that studying the peripheral circadian system is no straightforward matter. Comprehensive omics approaches are highly applicable to study organism-wide 24-hour rhythms in this complex system. They give a bird's-eye view of rhythmic processes, and make it possible to investigate how specific circumstances, for instance aging, influence these processes. In this thesis, a metabolomics approach was used to study rhythmic metabolites in the SCN and PVN (**chapter 6 & 8**) and in peripheral tissues (**chapter 7 & 8**). Moreover, the metabolic profiles of young and old mice from the SCN, PVN, liver, plasma and adipose tissue was compared to study how circadian rhythms change with aging (**chapter 7 & 8**).

In **chapter 6** we report on the development of a method to sample the SCN and PVN, two small brain areas in the hypothalamus. The usage of a punch ensured the same volume of the samples, to compare the concentrations of metabolites measured with mass spectrometry (MS) within those samples. We show that increasing neuronal activity in SCN or PVN tissue slices in middle of the night (with increased extracellular K^+ levels), leads to a rise in energy metabolites that are part of the Krebs cycle. In the intact animal, neuronal activity in the SCN is low at night, and increases when the animal is exposed to light. The neuronal response to a light pulse triggers a well-known signaling cascade, which results in either a phase advance or delay of circadian rhythms, from gene expression to behavior. Apart from providing energy, the intermediates of the Krebs cycle, and other metabolites can themselves act as signaling molecules and modify, e.g., receptor sensitivity, thereby contributing to the phase change in response to a light pulse (Martínez-Reyes & Chandel, 2020). For instance, it has recently been found that Krebs cycle intermediates – previously thought to be only by-products of cellular metabolism – are involved in signaling cascades controlling gene expression and protein remodeling (Martínez-Reyes & Chandel, 2020). This makes the study of metabolomics relevant beyond metabolic pathway analysis and biomarker identification.

In **chapter 7** we identified two metabolites which have the potential to be involved in currently unknown circadian signaling. Two amino acids, alpha-aminobutyric acid (AABA) and beta-aminoisobutyric acid (BAIBA) display interesting rhythms in the brain and periphery, which raises the question whether these metabolites could have a function

in the circadian system. BAIBA was recently discovered to be involve in the browning of white adipose tissue (Roberts *et al.*, 2014). Because of the metabolic advantages of brown fat, this discovery has boosted the research interest in BAIBA. BAIBA has been found to increase leptin secretion by WAT, likely through β-hydroxybutyrate, and induces fatty acid oxidation, the process of breaking down fatty acids in mitochondria (Begriche *et al.*, 2010). In **chapter 7** we found that BAIBA levels peak at the beginning of the day in the liver, which coincides with the peak in fatty acids with shorter acyl chains we observed in **chapter 8** in adipose tissue. Unfortunately, BAIBA could not be reliably measured in plasma, which prevents further speculation about its function in the periphery. The only known effect of BAIBA in the brain is inhibiting neuroinflammation, caused by a high-fat diet in mice [REF?]. However, the fact that BAIBA is rhythmic in both the SCN and PVN in young and in old mice, and correlates significantly between the SCN and PVN in young mice, raises the possibility of an important function in these brain areas. Moreover, there might be a link to the clock, through the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). PGC-1α is a transcriptional coactivator for genes with a role in energy metabolism. In myocytes, overexpression of PGC-1α stimulates the expression of BAIBA (Roberts *et al.*, 2014). PGC-1α is rhythmically expressed in the liver and muscle, and influences the expression of the core clock genes BMAL1 and CLOCK. The second interesting amino acid, AABA, we found to be rhythmic in the SCN, PVN and plasma, and likely also in the liver of young mice. Moreover, AABA levels are significantly correlated between the liver and plasma, and to a lesser extend also between the SCN and the other investigated tissues. Unfortunately, little is known about the role of AABA in the body; however, this metabolite does have a link to several metabolic and hepatic diseases, as well as neurological disorders (Fonteh *et al.*, 2007; Cheng *et al.*, 2012; Yang *et al.*, 2013). Both BAIBA and AABA have the potential to be relevant players in circadian rhythms and metabolism.

In **chapter 8** we explored patterns in lipid content of white and brown adipose tissue. We found that both in WAT and BAT a little over 20% of lipids fluctuate in abundance over the day. In WAT, most rhythmic lipids belong to the group of triacylglycerols (TAGs), followed by alkyldiacylglycerols (TG[O]s). Metabolites in both groups peak typically at the end of the dark phase in young mice and likely reflect dietary uptake of lipids. In BAT, the majority of rhythmic lipids belong to TAGs and Diacylglycerols (DAGs). Contrary to WAT, a significant part of these lipids peak during the day. Approximately half of the DAGs reach their maximum level in the light period (around ZT6-8), whereas a small portion of the TAGs peak around ZT 8-10. The lipids that reach the highest level during the light phase are lipids with acyl chain lengths longer than C18:x, which is likely a consequence of fatty acid elongation, and incorporation in lipids, as this coincides with a peak in expression of enzymes involved in de novo fatty acid synthesis and elongation in the liver (Hatori *et al.*, 2012; Greenwell *et al.*, 2019). Another group of lipids with a large percentage of

rhythmic profiles were phosphoinositides (PIs). In both BAT and WAT approximately 20% of the measured PI levels fluctuated over the day, which is similar to results from studies on muscle and liver tissue (Aviram *et al.*, 2016; Loizides-Mangold *et al.*, 2017). PIs are part of the cell membrane, but more importantly, they are involved in signaling pathways that regulate the cell cycle and energy metabolism (Hirsch *et al.*, 2020). We found that most lipids peak at the dark phase, which we also observed for amines in **chapter 7**. In the liver, most amines peak around ZT16, in plasma around ZT22. The peak times we found in our study for amines in the liver correspond well with results from Eckel-Mahan et al. (2013). In addition, this study found that for 5 out of 6 investigated classes of metabolites, most metabolites peak during the night-time. Since this corresponds with the period of the light-dark cycle that mice are active, it is likely related to food intake and higher metabolism. Indeed, it has been shown that feeding and fasting rhythms affect metabolite levels (Greco *et al.*, 2021). Therefore, it is worthwhile to design experiments using different feeding regimes, for instance a (short) period of fasting before tissue collection (see chapter 6), enabling the separation of the effect of feeding rhythms from endogenous rhythms.

1.3 Multi-level effect of aging on the circadian system

The aging process causes changes in the stability and plasticity of the circadian system, which is noticeable in the increased difficulties to adapt to shift work with aging. Also, there is a clear association between age and chronotype: aging in humans is accompanied with increased morningness (Roenneberg *et al.*, 2007). Moreover, many of the elderly experience the debilitating effects of fragmented sleep, as many feel tired and sleepy during the day, but have trouble sleeping well at night (Carskadon *et al.*, 1982). Therefore, investigating the age-related changes in circadian clock function and understanding the underlying mechanisms are important. It will identify the parts of the circadian system most vulnerable to aging, but also shows components reaching a new stable balance and finally point to potential targets for restoration of old clocks.

In **chapter 2** of this thesis we discussed recent advances in the knowledge of the circadian system in aging, and potential targets for interventions. One of the main conclusions is that aging does not affect all parts of the circadian system equally. This is substantiated by the research described in **chapter 5**, showing that PER2 expression rhythms are similar in the SCN of young and old mice under a 12:12 light-dark cycle. However, in the same mice, behavioral rhythms are significantly affected by age. This research is part of a growing body of literature, listed in **chapter 2**, reporting a minimal effect of aging on the molecular clock, while most other evaluated features of the SCN, and other parts of the circadian system clearly deteriorate with aging. For instance, on the level of the SCN, there are clear changes in electrical activity (Satinoff *et al.*, 1993; Nakamura *et al.*, 2011) and neuropeptide expression (Roozendaal *et al.*, 1987; Chee *et al.*, 1988; Pereira *et al.*, 2005) related to aging.

In addition, in **chapter 8** we found a significant effect of aging on levels and daily rhythms in metabolites, and the relationship of metabolite levels between tissues.

In this thesis we show that there are clear differences between tissues in aging-associated decline of the circadian system, which will likely be the case for many more features of the circadian system. This highlights the importance of a multi-level approach in studying the aging circadian system, and is also relevant for the development of therapeutic interventions. The application of a combination of interventions that target both the SCN, with for example light exposure, as well as peripheral clocks, with for example timed meals and/or physical activity, could boost the circadian system and prevent or restore damage from a malfunctioning circadian system, leading to overall better health.

1.4 Concluding remarks and further directions

In this thesis our studies of the circadian system – from molecules to behavior and from brain to body – are presented. We have shown that the SCN network adjusts to photoperiod length and that these adjustments are not the same throughout the SCN. While the capacity of the molecular clock to adjust to photoperiod does not change markedly in old age, the behavioral adaptation does deteriorate during aging. Aging is also accompanied by moderate to severe changes in metabolite oscillations in the central pacemaker and in the periphery. However, most striking is the complete loss of correlations of metabolite levels between tissues.

We know that the maintenance as well as adaptation of circadian rhythms to changing circumstances are important for the healthy functioning of an organism. A deterioration of circadian rhythms can become a downward spiral, affecting the whole-body homeostasis. Maintaining proper circadian rhythmicity therefore seems important for, among others, healthy aging. However, it is challenging to study the effect of disturbed circadian rhythms and determine direct causal relations, given the complex – often bidirectional – interactions of the circadian system. The assessments of the circadian system at multiple levels within individual animals gave us the opportunity to study their interconnectedness. By studying these connections within both young and old animals, we took a first step towards evaluating how aging affects the interactions between the different levels of the circadian system.

A better understanding of the interplay between the SCN and other clocks within the circadian system, and how their coherence is affected by, for example, aging will be relevant to advance the treatment of disturbed circadian rhythms. A better treatment will benefit overall health and will improve quality of life in the elderly. Metabolomics, especially when combined with other research tools, is already proving to be very useful to evaluate the system-wide circadian coherence (Dallmann *et al.*, 2012; Patel *et al.*, 2012;

Dyar *et al.*, 2018). It has the potential to identify targets for chronotherapy as well as to test the effect and efficacy of therapeutical interventions.

It is likely that a combined approach, targeting multiple levels of the circadian system, will be most beneficial. Adequate light exposure during the day, food and activity at the right, limited time of the day are easy to implement and will improve healthy aging and overall wellbeing. The task at hand is to specify the parameters and identify new targets to optimize the effect of chronotherapy on our health.

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