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### The circadian system

*A regulatory feedback network of periphery and brain*

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## CHAPTER 7 - GENERAL DISCUSSION

Based upon: The circadian system: A regulatory feedback network of periphery and brain.

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7

## **Abstract**

Circadian rhythms are generated by the autonomous circadian master clock in the suprachiasmatic nucleus (SCN) and by clock genes that are present in all tissues of the body. The SCN times these peripheral clocks, as well as behavioral and physiological processes. Recent studies show that frequent violations of conditions set by our biological clock, as a consequence of shift work, jet lag, sleep deprivation or simply eating at the wrong time of the day, may have deleterious effects on health. This infringement, also known as circadian desynchronization, is associated with chronic diseases like diabetes, hypertension, cancer and psychiatric disorders. In this discussion we will evaluate the main hypothesis of this thesis: “The SCN is not solely an autonomous master clock imposing its rhythm onto the periphery, but depends on peripheral feedback in order to effectively regulate physiological functions”. We relate our present results to recent evidence showing that the SCN depends on peripheral feedback to fine-tune its output and adjust physiological processes to the requirements of the moment. This feedback can vary from neuronal or hormonal signals from the liver to, as we show, changes in blood pressure. Desynchronization between the SCN, light input and peripheral signals, potentially renders the circadian network dysfunctional, resulting in a breakdown of many physiological functions that are coordinated by the SCN. This disrupts core clock rhythms in the periphery and disorganizes cellular processes normally driven by the synchrony between behavior and peripheral signals, and neuronal and humoral output of the hypothalamus. Consequently this thesis proposes that the loss of synchrony between the different elements of this circadian network during shiftwork and (social) jet lag is the reason for the occurrence of many health problems.

## From circadian synchronization and balance to divergence and disease

### *Synchronizing the cardiovascular system*

Cardiovascular incidents follow a daily rhythm that has its highest incidence early in the activity period (Muller and others, 1985) suggesting the involvement of the circadian system in cardiovascular pathology. Recent studies have also emphasized a role for peripheral clock genes in cellular processes associated with blood pressure (BP) control. Within the adrenal, absence of *cry* has been associated with hyperaldosteronism and hypertension (Doi and others, 2010). In the kidney, the absence of *per1* was associated with the deregulation of renal epithelial sodium channels (Gumz and others, 2009). Other clock genes have been implicated in vascular endothelial function (Viswambharan and others, 2007; Cheng and others, 2011) or in thrombogenesis (Westgate and others, 2008) with potential relevance for humans (Scheer and others, 2011). Still, the prevention of vascular pathology is dependent on the integrity and rhythmicity of the circadian system as a whole and not the mere consequence of *bmal1* deficiency or *clock* mutation alone (Gibbs and others, 2014).

Chronic changes in the SCN have been observed in both hypertensive humans and rats (Peters and others, 1994; Goncharuk and others, 2001) showing a link between an altered circadian system and disease. In **chapter two** we demonstrated the importance of the SCN not only as an autonomous clock, but also as an integration site in the physiological circuits regulating BP as is illustrated by the observation that the SCN receives cardiovascular feedback via the nucleus tractus solitarius (NTS). We showed that glutamatergic NTS projections to the SCN terminate in the ventrolateral part of the SCN, where light information also enters and BP elevations not only induced increased neuronal activity as measured by c-Fos in the NTS but also in the SCN. Lesioning the caudal NTS in turn prevented this activation. The increase of SCN neuronal activity by hypertensive stimuli suggested involvement of the SCN in counteracting BP elevations. As such we proved that the SCN is incorporated in a neuronal feedback circuit arising from the NTS, modulating cardiovascular reactivity. It also suggests that untimely changes in BP, which may occur in shift work, jet lag or prolonged activity at night, potentially disturbs the functionality of the SCN via this novel cardiovascular-NTS-SCN feedback pathway.

A mouse model of induced cardiac hypertrophy is illustrative for the involvement of the SCN in cardiovascular regulation and development of disease. Forced desynchronization through shortened light-dark cycles significantly increased cardiac pathology as compared to synchronized animals. Restoration of the natural daily rhythmicity, thus resynchronizing the SCN and periphery, fully reversed this pathophysiology (Martino and others, 2007). This suggests that circadian desynchrony can greatly contribute to the progression of organ dysfunction and development of disease, while restoration of circadian rhythmicity potently reverses pathology. In recent years chrono-pharmacology has thus developed into a potentially effective way of treating cardiovascular disease associated with circadian desynchronization; e.g. the treatment of “non-dipper”

hypertensive patients is more effective when the therapeutic window of anti-hypertensive drugs is aimed to match the physiological trough in blood pressure (Hermida and others, 2008). In addition, evening administration of low-dose aspirin significantly reduces morning platelet reactivity and thus the risk of thrombo-embolic events, which peak early in the morning (Bonten and others, 2015). Interestingly, repetitive night-time melatonin administration, known to amplify the rhythm of melatonin secretion via an action on the SCN (Bothorel and others, 2002), substantially reduces blood pressure in hypertensive patients (Scheer and others, 2004).

In conclusion, it can be proposed that synchrony between the cardiovascular system and the SCN, through our demonstrated cardiovascular-NTS-SCN feedback pathway, is essential for homeostasis, whereas desynchronization within this system could ultimately result in the development of cardiovascular disease.

#### *Circadian dysfunction in psychiatric disorders and their treatment*

People suffering from depression, bipolar disorder (Novakova and others, 2014), anxiety or schizophrenia (Wulff and others, 2010) exhibit fatigue, changes in sleep, appetite and body weight and circadian desynchronization. Patients exhibit dampened temperature rhythms (Avery and others, 1999), altered cortisol levels —itself a predictor for the course of illness— (Vreeburg and others, 2013) and melatonin secretion (Lewy and others, 2006). Other visible features of chronic circadian desynchronization associated with psychiatric disorders are metabolic syndrome, obesity, diabetes, hypertension and dyslipidemia (Vancampfort and others, 2015), all contributing to premature death occurring up to 10 years earlier as compared to the general population.

Clinically depressed individuals exhibit clock gene dysregulation in specific brain areas, abnormal phasing of clock gene expression and potentially disrupted phase relationships between individual circadian genes, suggesting a desynchronization within the circadian network (Li and others, 2013) This is supported by the observation that multiple simultaneous chronotherapeutic interventions aimed at synchronizing the circadian system, using bright light therapy and advancing the sleep phase, are an effective treatment for sustained improvement in severely depressed patients (Wu et al. 2009) which acts within 24-48 hours in 40%-60% of depressed patients. Conventional antidepressants usually require 2-8 weeks to meet response criteria. The delay, which may prolong suffering and increase suicidal risk, underlines the urgency of alternative treatment strategies. This study evaluates the combined efficacy of three established circadian-related treatments (SD, bright light [BL]. Interestingly, many pharmacological agents for the treatment of psychiatric disorders, i.e., olanzapine and quetiapine, have large cardiovascular and metabolic side effects that are under-diagnosed and undertreated (Steylen and others, 2013). Synchronizing the circadian system by administration of nightly melatonin, significantly decreases drug-induced proneness to obesity and blood pressure alterations (Romo-Nava and others, 2014). As such, in **chapter three** we demonstrated the SCN

as a key nucleus mediating the early effects of olanzapine on cardiovascular function. Second generation antipsychotics are associated with adverse cardio metabolic side effects contributing to premature mortality in patients. While mechanisms mediating these cardiometabolic side effects remain poorly understood, three independent studies (Romo-Nava, 2014; Modabbernia, 2014; Mostafavi, 2014) demonstrated that melatonin was protective against cardio-metabolic risk in patients receiving antipsychotics. We demonstrated that melatonin has an opposing and potentially protective effects on cardiovascular disease associated with olanzapine use. This finding re-affirms an important role of melatonin in synchronizing the circadian system and prevention of cardiovascular and metabolic pathology, also in relation to adverse effects associated with antipsychotic drugs.

*Metabolic information: multiple sources and multiple integration sites.*

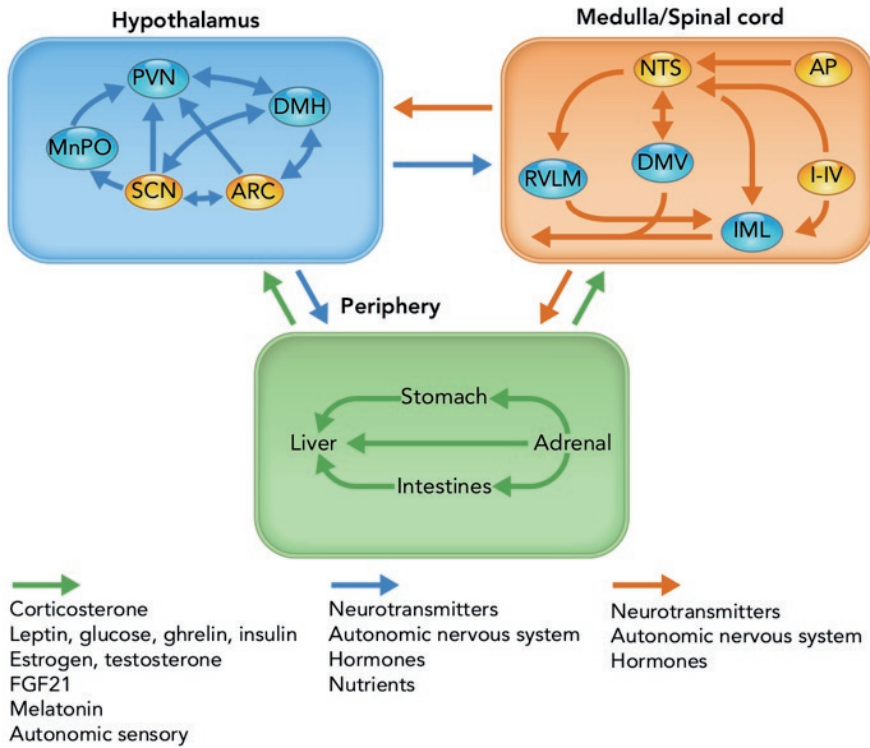
An optimal metabolic state is of such importance that almost all physiological systems react to metabolic cues. Since the availability of food supply is evolutionary closely linked to the activity period, the biological clock—in interaction with the hypothalamus—plays an essential role in timing adequate circadian metabolic control. This only recently has become clear by experiments showing that the SCN receives strong feedback of peripheral metabolic signals. For example, the liver is capable of sending a starvation signal by fibroblast growth factor-21 (FGF21) secretion into the circulation, directly reaching SCN receptors. This induces a decrease of systemic insulin, an increase of corticosterone levels, an inhibition of growth and a change in locomotor activity and reproduction (Bookout and others, 2013). Besides this direct metabolic feedback at the level of the SCN, in **chapter four and five** we discussed the intergeniculate leaflet (IGL) and arcuate nucleus (ARC) respectively as important relays in transmitting metabolic feedback to the SCN. The ARC is the main metabolic integration center of the hypothalamus. For example, leptin, secreted by adipose tissue, may target the SCN via the ARC since ablation of leptin receptor expressing neurons in the ARC leads to the disruption of the circadian rhythm in food intake (Li and others, 2012). Similarly, deletion of ROCK1, a key kinase in the signaling of leptin, leads to severe diminishment of spontaneous daily locomotor activity, suggesting an essential role for metabolic feedback to the ARC in maintaining circadian rhythmicity (Li and others, 2012; Huang and others, 2012). Moreover, circadian control of body temperature is dependent on concurring arginine vasopressin (AVP) and  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) signaling from SCN and ARC to the medial preoptic area (MnPO), orchestrating a time-dependent temperature decrease (Guzman-Ruiz and others, 2015). This illustrates the need for the SCN to synchronize via metabolic cues in order to obtain adequate control of physiology. These metabolic cues are also potentially important for rhythmicity as illustrated by observations that metabolic signals originating from the ARC (Yi and others, 2008), lateral hypothalamus (LH) (Belle and others, 2014) or IGL are capable of changing the activity of the ventrolateral area of the SCN.

Proving the latter, in **chapter four**, we show that the IGL–SCN axis is also importantly changed by the metabolic condition of an animal and that NPY and gamma-aminobutyric acid (GABA) have a prominent role in transmitting this metabolic information. As such we prove that the SCN does not depend exclusively on the ARC or NTS to obtain information about the metabolic status, but that the IGL can also transmit metabolic information that it receives from NTS and the nucleus gracilis. This integration of metabolic information within the ventrolateral area of the SCN, associated with synchronizing the rhythm of SCN activity, could provide a pathway for the synchronizing effect of food on the SCN. This, for example, was shown in hypocaloric food restricted animals whereby, in contrast to normocaloric fed animals, feeding cues were able to alter SCN clock gene oscillations (Mendoza and others, 2005).

*In vivo* lesioning studies have demonstrated the importance of the network properties of the mediobasal hypothalamus in maintaining circadian rhythmicity (Gerkema and others, 1990), thus confirming early studies showing loss of activity rhythms due to knife cuts posterior to the SCN (Moore and Eichler, 1972). Altogether this argues for a system where the SCN, coupled to other hypothalamic nuclei (and peripheral organs), forms a network of oscillators, essential for maintaining circadian rhythmicity.

This may explain why long-term desynchronous metabolic feedback has a deleterious effect on the circadian system and on health. In rodents, high fat diet or food intake during the rest phase desynchronizes and dampens clock gene rhythmicity (Damiola and others, 2000), leading to obesity, insulin resistance (Dibner and Schibler, 2015) and cardiovascular disease (Paschos and FitzGerald, 2010). Interestingly, when feeding mice high-fat diet (HFD) in a time restricted manner they were protected from obesity, hyperinsulinemia, hepatic steatosis, and inflammation as compared to their *ad libitum* counterparts, while consuming an equivalent amount of calories (Hatori et al. 2012) animals fed a high-fat diet (HFD). These observations provide a link as to why these diseases, including cancer, have an increased incidence in shift workers (Davis and others, 2001; Knutsson and Kempe, 2014). Another example can be found in humans with night-eating syndrome, where high caloric intake during the resting phase disrupts the normal circadian pattern and results in an increased tendency to develop obesity (Howell and Schenck, 2009). For a detailed review of metabolic desynchronization and consequential health effects, see (Gamble and others, 2013).

Since behavior and SCN clock genes can also be synchronized to food (Lamont and others, 2005; Mendoza and others, 2005), food may have a protective effect on desynchrony. This is shown in a rat model of shift work, whereby restricting food intake to the normal activity period while working in the rest period induces a significantly lower weight gain and increased insulin sensitivity as compared to *ad libitum* feeding shift worker animals (Salgado-Delgado and others, 2010). This shows that the hypothalamic circadian system, with the SCN at its core, is a complex reciprocally connected network that organizes metabolic homeostasis of the body and is capable of being (de)synchronized through peripheral signals (Figure 1). In the next paragraphs we will give some examples of how SCN driven physiological rhythms are not driven in isolation but depend on each other in order to become fully rhythmic.



**Figure 1. Feedback networks of the circadian system.** Our hypothesis on the functioning of the circadian system consists of multiple inter-connected feedback loops regulating physiology. Illustrated are three interconnected feedback loops: hypothalamus, brain stem/spinal cord, and periphery, within which, of course, are many other feedback loops on cellular, tissue, and organ level. 1) Within the hypothalamus, the suprachiasmatic nucleus (SCN) sends timing signals to several target areas including the medial preoptic area (MnPO) for temperature regulation and reproduction; paraventricular nucleus (PVN) for hormone release and autonomic output; dorsomedial nucleus of the hypothalamus (DMH) as hypothalamic integration center; arcuate nucleus (ARC) as center for sensory metabolic information. All these nuclei are interconnected and the SCN receives direct feedback from all but the PVN. 2) The brain stem/spinal cord feedback loop receives direct and indirect temporal information through the rostral ventral lateral medulla (RVLM), nucleus tractus solitarius (NTS), area postrema (AP), and the sensory layers lamina I–IV (I–IV) of the spinal cord. These nuclei function as integration centers for peripheral and central signals and are responsible for autonomic physiological reflexes transmitted to the dorsal motor nucleus of the vagus (DMV) and intermediolateral column (IML) that serve as autonomic output nuclei. 3) The periphery receives temporal signals from the hypothalamus via autonomic output of the parasympathetic motor neurons in the DMV and via sympathetic motoneurons in the IML. In addition, circadian signals are also transmitted via hormones such as melatonin and corticosterone or by nutrients like glucose. The red ovals represent structures that receive autonomic sensory feedback such as the area postrema (AP), NTS, and the sensory layers lamina I–IV (I–IV) of the spinal cord; or hormonal and metabolic feedback from the circulation such as the AP, ARC, and SCN. Moreover, peripheral organs may communicate with each other via a circuit consisting of autonomic sensory signaling to the AP, NTS, and I–IV of the spinal cord followed by reflex automatic adjustment of autonomic output. Any disturbance or desynchrony between and within these circuits could, in time, potentially lead to pathology and disease.

*Temperature: Circadian and metabolic influences*

Ahead of the active phase, core body temperature (T<sub>b</sub>) starts to increase, independent of locomotor activity, while T<sub>b</sub> drops just prior to activity cessation in the resting phase (Refinetti and Menaker, 1992; Scheer and others, 2005). The central role of the SCN in the metabolic and temperature regulating network becomes clear when noting that SCN lesions prevent not only temporal T<sub>b</sub> rhythmicity but also fasting-induced T<sub>b</sub> decrease (Liu and others, 2002). The fasting-induced decrease in body temperature is preceded by a drop in metabolic rate hinting at a significant role for the ARC in this regulatory system. In fact, numerous hypothalamic nuclei, i.e., dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), ARC and MnPO with the SCN at its core, are all jointly involved in temperature control (Morrison and Nakamura, 2011), revealing a complex temperature-regulating network. On the basis of observed interactions between the SCN and ARC (Saeb-Parsy and Dyball, 2003; Guzman-Ruiz and others, 2014) it was shown that the T<sub>b</sub> rhythm depends on an interplay between temporal signals from the SCN and metabolic signals arising from the ARC (Guzman-Ruiz and others, 2015). Not only is an SCN driven rhythm of ARC neurons essential for this, it also requires a synchronized release of SCN vasopressin and ARC  $\alpha$ -MSH neurotransmitters in the MnPO, to organize diurnal temperature decreases in rats. Lesions of specific ARC neuronal populations critically modify circadian patterns in food intake and locomotor activity (Coppari and others, 2005; Wiater and others, 2011; Li and others, 2012; Huang et al 2013) Further exploring this notion, in **chapter five** we showed that the SCN-ARC axis serves to synchronize SCN and ARC output and that circadian rhythms in activity, temperature and corticosterone are lost in constant dark conditions when this SCN-ARC axis is disrupted. We also demonstrate that metabolic feedback to the SCN is significantly altered when SCN-ARC interconnectivity is cut causing an altered neuronal activation of the ARC but also of the SCN. This confirms the previously suggested idea (Webb et al., 2009; Hu et al., 2012; Buijs et al., 2016), that the SCN functions inside a larger circadian network of tightly linked oscillatory feedback circuits whose integral function is essential for regulating physiologic and behavioral functions. Long-term desynchronization within this circadian network due to changes in dietary habits, chronic jetlag, or shift work is known to contribute to pathology associated with “modern lifestyle,” such as hypertension, obesity, diabetes, and cancer (Scheer et al., 2010; Leproult et al., 2014; Kettner et al., 2016). We therefore propose that ill-timed food intake and altered metabolic signals are able to alter normal ARC activity patterns, as such changing its synchronization with the SCN and as consequence disrupting associated behavioral and hormonal patterns. Thus, faulty network connections or erroneous feedback may reshape the circadian system to a new equilibrium, leading to physiologic impairment and pathology.

*The hypothalamic-pituitary-adrenal axis and the preparation for activity and food*

The hypothalamic-pituitary-adrenal (HPA) axis is under strong control of the SCN. SCN induced release of vasopressin in the beginning of the sleep phase has a strong inhibitory influence on the secretion of ACTH and corticosterone (Kalsbeek and others, 1996) whereas diminishing this inhibitory input towards the beginning of the active period induces the diurnal peak in corticosterone preparing the animals for activity onset. Interestingly, crepuscular animals, active at dusk and dawn, have two SCN driven peaks of corticosterone (Kalsbeek and others, 2008). Closely associated with, but not driven by corticosterone, is the peak of circulating glucose. Corticosterone signaling to the ARC reduces hepatic insulin sensitivity (Yi and others, 2012), creating a perfect harmony between the corticosterone and glucose peaks, whose rhythms are synchronized by the SCN. This observation might be relevant in explaining why such strong metabolic alterations are observed in hypercortisolism. The same is seen in stress disorders or in chronic jet-lag/shift work mimicking the effects of chronic stress, causing increased glucocorticoid production which is correlated with developing diabetes and obesity (Kolbe, Dumbell, and Oster 2015).

*Locomotor activity fine-tunes SCN rhythmicity*

Locomotor activity, closely associated with arousal, has an effect on SCN neuronal activity and synchronization of the circadian system, although at a much lower intensity than light. Nevertheless, activity has been shown to directly inhibit the neuronal firing in the SCN, especially during the subjective day (Yamazaki and others, 1998; Schaap and Meijer, 2001). Thus activity may be a potential (de)synchronizer of the circadian system. The synchronizing property is seen in daily forced locomotor activity in constant darkness (DD): when the forced activity is halted, free running rhythms run closer to 24-h than the prior basal free running rhythm, even in mice that lack VIP receptor type 2 (VPAC2R) (Hughes and Piggins, 2012). VIP and its receptor, VPAC2R, are important for synchronizing the SCN. As such animals lacking VPAC2R show changed circadian rhythms and impaired synchronization to light cues. This suggests a form of synchronization occurring following daily forced locomotor activity. Activity may also feed back on areas outside the SCN, for example, the Raphe nucleus is known to be important for the synchronization of SCN neuronal activity through its serotonin projections, i.e., by desensitizing the SCN to light (Van de Kar and Lorens, 1979; Malek and others, 2007). The SCN and hypothalamus take turns to synchronize the Raphe nucleus through locomotor activity and corticosterone; they both target and induce rhythmicity in serotonin synthesis (Malek and others, 2007). This is significant because it illustrates how the SCN receives feedback related to its own output and is able to synchronize through locomotor activity and corticosterone release, serotonin synthesis. Another example is melatonin secretion, driven by the SCN at night, it also enforces the night signal through melatonin receptors in the SCN (Reppert, 1997; van den Top et al. 2001; Bothorel and others, 2002). These examples illustrate how important amplification of the proper circadian rhythms can be for maintaining or restoring adequate physiological function.

### *Synchronizing the immune system*

The circadian system has a strong influence on the immune system, e.g., mortality is greater when bacterial endotoxin, LPS (lipopolysaccharide), is given to rodents during the night, a time that coincides with increased pro-inflammatory cytokine production after LPS (Marpegan and others, 2005). It is suggested that the SCN is incorporated into a regulatory circuit between the immune system and the brain, as shown by the activation of the SCN following an inflammatory stimulus. Ablation of the SCN amplifies the innate immune response several fold, suggesting an inhibitory influence of the SCN (Guerrero-Vargas and others, 2014). Clock genes in immune cells also play an important role in the immune response (Silver and others, 2012), further emphasizing the role of the circadian system. Cytokine interferon- $\alpha$ , used in cancer treatment, has a strong disruptive effect on locomotor activity and body temperature as well as on clock gene expression in the SCN. These adverse effects are for a large part prevented by changing the time of administration (Ohdo and others, 2001), emphasizing the strong interaction between circadian regulation and the immune system. Circadian desynchronization induced by shift work in rats is associated with an enhanced inflammatory response that was prevented by synchronizing food with the normal feeding time (Guerrero-Vargas and others, 2015). For that reason, therapies limiting food intake to the normal activity period may help to balance the immune response and may prevent development of inflammatory diseases.

Since the discovery of oscillatory clock gene expression in tumors, chrono-pharmacological cancer treatment—finding the optimal times for drug administration based on circadian variation in drug pharmacokinetics, efficacy and tolerance—has received much attention, and has given promising results. Studies show that chrono-chemotherapy improved therapeutic outcome and survival for numerous types of cancer in humans (Innominato and others, 2014).

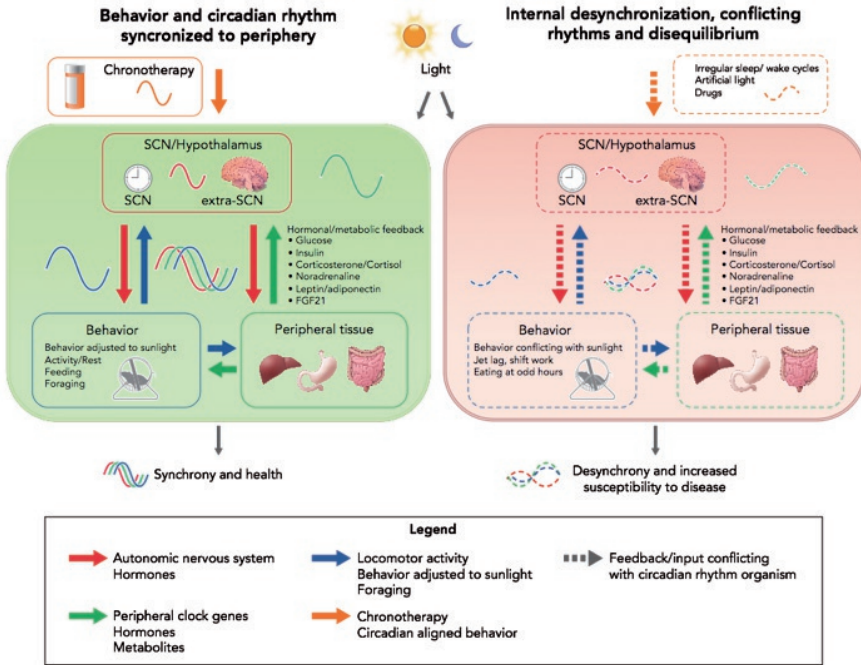
### *Aligning the reproductive system*

The SCN is essential for integrating and synchronizing all neuroendocrine signals involved in initiating a well-timed GnRH–LH surge (Smarr and others, 2012). Several studies have shown the importance of SCN signaling, through VIP (Van Der Beek and others, 1997; Sun and others, 2012) directed to GnRH neurons, or vasopressin (Palm and others, 1999), in interaction with the Kisspeptin system (Smarr and others, 2012), for accurately timing the LH surge. However, reproduction does not solely depend on a correctly functioning SCN: without peripheral signals, i.e. about the metabolic state of the body, a reproductive cycle cannot be completed. A liver-neuroendocrine signaling pathway has recently been described through which FGF21, a fasting-induced hepatokine, acts through the SCN, suppressing the vasopressin-kisspeptin signaling cascade and thereby inhibiting ovulation during starvation (Owen and others, 2013). Other fasting-elicited hormonal changes, such as low leptin levels, also prevent a successful cycle (Bellefontaine and others, 2014). These examples show that not only circadian timing but also synchronized metabolic and

physiological feedback is essential for a successful reproductive cycle. In **chapter six** we demonstrated the importance of reciprocal interaction between the SCN, the inhibitory RFamide-related peptide-3 (RFRP-3) neurons (the mammalian ortholog of gonadotropin-inhibitory hormone), the stimulatory Kisspeptin neurons in the AVPV and the Kisspeptin neurons in the ARC (that are probably both stimulatory and inhibitory) for a successful LH surge. This indicates the presence of another integrated feedback/feedforward circuits acting in synchrony for the adequate timing of physiological events.

## Conclusions

In this thesis we have argued that, with the SCN at its center, the circadian system forms a coupled multi-oscillatory system, wherein each participating nucleus receives a multitude of signals, providing this information into the system, thus fine-tuning circadian rhythmicity. The oscillatory organization of this system is maintained through the core rhythm of the SCN that, through hormonal, neuronal or behavioral signals, fine-tunes bodily functions to the activity or resting period. The autonomous rhythm of the SCN is augmented and fortified through cerebral and peripheral feedback, making the circadian system more robust and less prone to environmental variations. However, as a consequence long-term perturbations through drug use, untimely light exposure or disorderly behavior will induce peripheral signaling capable of disrupting this harmony, rendering an individual more in disbalance and therefore susceptible to disease. The SCN not only functions as a sophisticated timing mechanism but is integrated in multiple oscillatory feedback circuits involved in the regulation of physiological and behavioral functions. The proneness of oscillatory networks to desynchronization has recently been analyzed through a mathematical model of the evolution of feedback networks in bacteria, fungi and drosophila (Noman and others, 2015). The robustness of a network was demonstrated to be dependent on the number of interconnections and the number of regulators per connection, with an increasing number of interconnections and regulators associated with an increase in robustness. This is also illustrative for the circadian systems functioning, e.g., by the molecular feedback loops controlling clock genes rhythmicity inside individual SCN neurons. In turn, these weakly rhythmic individual neurons (Herzog and Schwartz, 2002) function inside larger coupled network, making up the SCN, driving a common rhythm and regulating its circadian output. Considering the studies presented and/or reviewed in this thesis, the SCN is in turn incorporated in a larger hypothalamic network of oscillators integrating peripheral signals. Finally, behavior and external stimuli like food intake or drug (ab)use also have their place in the feedback circuitry of the organism, adjusting adequate circadian functions (Figure 2). The multiple intertwined feedback loops of the circadian system makes it robust and capable of withstanding brief erroneous feedback, but months or years of conflicting feedback, ill-timed behavior or chronic jetlag/shift work, will increase susceptibility to pathology and disease.



**Figure 2. Proposed organization of synchrony in the circadian system.** Whether an organism is in equilibrium depends on whether the multitude of circadian rhythms expressed are in synchrony or oppose each other. A synchronized and healthy situation is depicted on the *left* where light synchronizes the activity and rhythm of the SCN. The SCN transmits this rhythm via the autonomic nervous system (ANS), hormone secretion, and behavior to the body, thus synchronizing the periphery and adjusting the physiology according to time of day (red arrow). In turn, the periphery sends feedback to the brain via metabolites, hormones, and autonomic sensory pathways (green arrow). The periphery, through release of hormones and metabolites and in concert with autonomic signaling, also affects locomotor activity and foraging behavior (green arrow). Behavior, through locomotor activity or eating behavior, feeds back to the periphery and the brain (blue arrows), amplifying circadian rhythmicity and synchrony. When, as depicted on the *right*, the light-dark cycle, behavior, and peripheral signals do not align with that of the SCN or the hypothalamus (broken arrows), the deleterious feedback interferes with the circadian system equilibrium, which in the long term could potentially lead to desynchrony and development of disease. Chronotherapy in the form of circadian-timed drug administration or synchronizing sleeping/eating behavior with the light-dark cycle (orange arrow) can augment circadian system resynchronization, potentially reversing pathology and reducing disease.

## Future directions

The main goal of the present thesis was to establish the significance of a circadian feedback network with the SCN at its core. We have tried to reveal feedback loops incorporating the SCN and demonstrate its ability to affect SCN activity resulting in altered physiological regulation. The complex nature of our proposed circadian network suggests that it will take time before the full complexity of the circadian system will be understood. We and others (Brandstaetter, 2004), suggest that a holistic approach will be crucial in filling in the many gaps in our knowledge of the circadian system. Many current developments in (molecular) chronobiology, such as *in vitro* analysis, conditional knock-out animals and optogenetics are doubtlessly invaluable and indispensable in present chronobiology research. However, considering the complexity of the circadian network, caution should be exercised in extrapolating conclusions from *in vitro* analysis into *in vivo* models. For example, in spite of *in vitro* data suggesting the direct production of NAMPT via CLOCK/BMAL1 (Ramsey and others, 2009) it has been observed that in animals eating during the light period, NAD<sup>+</sup> and NAMPT, together with certain metabolic genes, do not follow the inversion of rhythm in core clock genes like CLOCK/BMAL1 (Salgado-Delgado and others, 2013). These observations indicate that *in vivo*, alternative essential molecular relationships prevail, likely driven by other components of the circadian system, such as melatonin or corticosterone. Testing isolated brain areas *in vitro*, or selectively activating small populations of neurons *in vivo* through optogenetics, gives insight into an isolated stimulus response, but fails to give a full picture as to how systemic physiological processes are truly regulated. Basic physiological experimentation and research is thus still very important for an understanding of physiological functions of the organism as a whole.

When we increase our understanding the complexity of physiological function and the entwinement of the circadian system with body functions, we will better be able to understand the adaptive changes taking place when deleterious feedback results in pathophysiological conditions and disease. As discussed, ultimately this understanding will help us to uncover new therapeutic strategies for e.g. cancer, obesity, cardiovascular disease and dementia.

Important progress has been made in understanding the impact of chronotherapy, especially in cancer (Ballesta et al. 2017). However, chronotherapy in clinical trials or in the hospital setting is still highly underused (Selfridge et al. 2016) physiological, and behavioral processes that oscillate in a 24-h cycle and can be entrained by external cues. Circadian clock molecules are responsible for the expression of regulatory components that modulate, among others, the cell's metabolism and energy consumption. In clinical practice, the regulation of clock mechanisms is relevant to biotransformation of therapeutics. Accordingly, xenobiotic metabolism and detoxification, the two processes that directly influence drug effectiveness and toxicity, are direct manifestations of the

daily oscillations of the cellular and biochemical processes taking place within the gastrointestinal, hepatic/biliary, and renal/urologic systems. Consequently, the impact of circadian timing should be factored in when developing therapeutic regimens aimed at achieving maximum efficacy, minimum toxicity, and decreased adverse effects in a patient. However, and despite a strong mechanistic foundation, only 0.16 % of ongoing clinical trials worldwide exploit the concept of 'time-of-day' administration to develop safer and more effective therapies. In this article, we (1. A more broad application of circadian timing should be made in developing new therapeutic strategies with a focus on maximizing efficacy, reducing toxicity, and decreasing adverse effects in patients. The future will be bright when chronotherapy is integrated in the development of more effective treatment paradigms.



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