Circadian timekeeping: from basic clock function to implications for health

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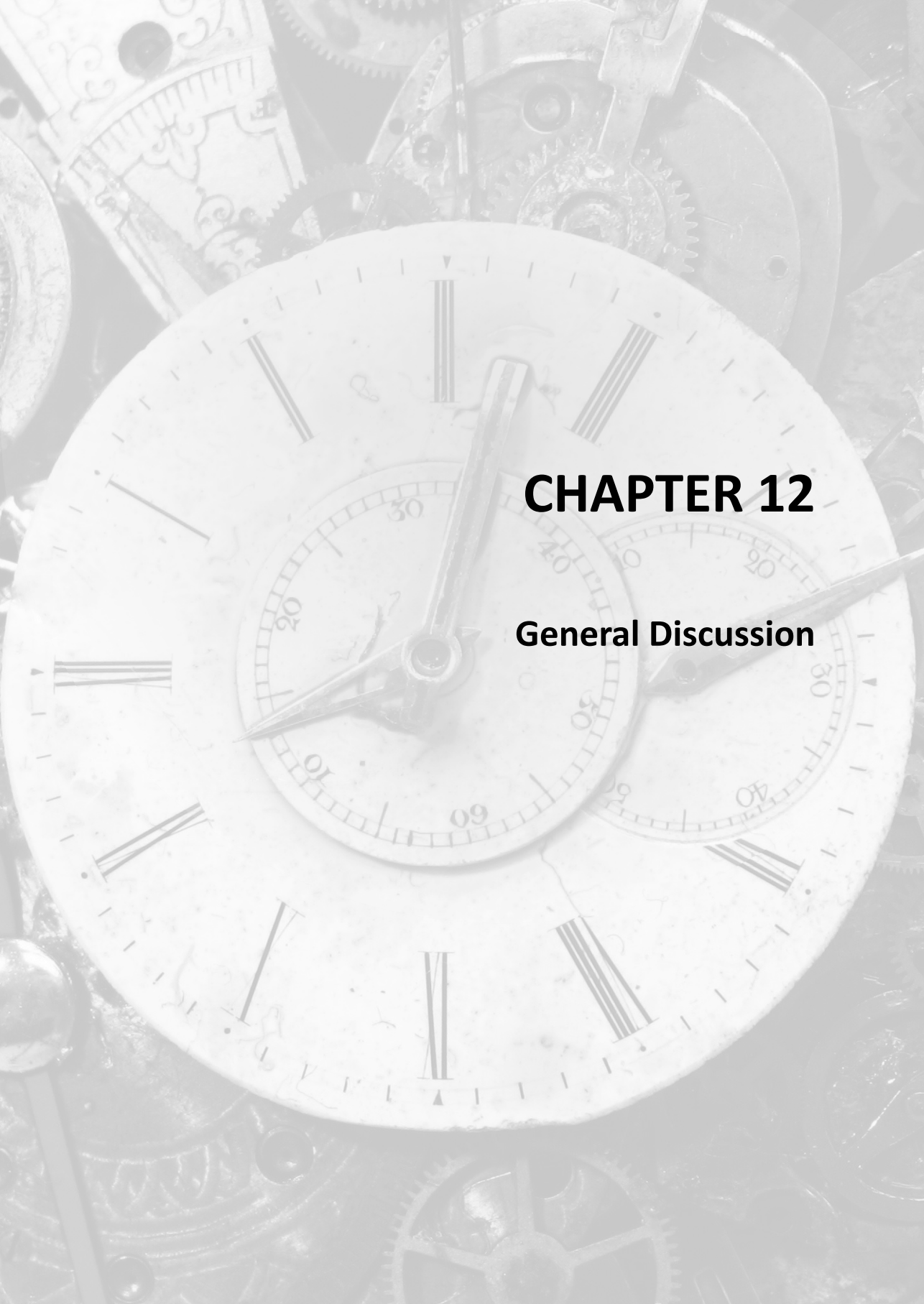

CHAPTER 12

General Discussion

Preface

Our body clock resides in the suprachiasmatic nuclei (SCN), a pinhead-sized area in the anterior hypothalamus that is responsible for synchronizing circadian rhythms to the external environment. It additionally coordinates temporal synchrony within the body, assures that our bodily physiology is optimally adjusted to our sleep-wake rhythm and prepares the body for states fitting the time of day. Disruption of this complex temporal physiology affects health. A deeper understanding of the functioning of the circadian system will help us understand these health effects and may provide opportunities to enhance fitness. Therefore, I will first describe how appropriate behavioral (**Chapter 6**) and photic input (**Chapter 4**) and strong synchrony amongst SCN neurons (**Chapter 4**) can provide robustness to the circadian system and how phase shifting capacity, important for entrainment, is influenced by sleep and caffeine (**Chapter 5**). Subsequently, I will discuss the rhythmic characteristics of animal models of circadian disruption that were used in studies described in **Chapter 7** and **Chapter 8**, and of human variations in temporal preferences (**Chapter 9**). Furthermore, I will examine the relationship between sleep and circadian rhythms and the contribution of detrimental sleep in models for circadian disruption. I will discuss the results of animal studies of **Chapter 7** and **Chapter 8** of disrupted circadian rhythms on metabolism and frailty parameters and discuss their applicability to humans. Specifically, I will compare the circadian states seen in constant light (LL, **Chapter 8**) and in aging. I will discuss the associations between chronotype and metabolic parameters (**Chapter 9**) and neurocognitive function (**Chapter 10**) in short sleeping obese individuals. I will discuss reversibility of adverse health parameters (**Chapter 8**, **Chapter 10**) and end with the clinical implications of findings in the current thesis.

Vasoactive intestinal peptide is necessary for seasonal rhythmicity

Studies in **Chapter 4** examine the function of VIP, a neurotransmitter implicated as an important coupling factor of SCN neurons. We demonstrate that the neurotransmitter VIP is necessary for *memory* of the environmental day length. Behavioral and neuronal activity patterns in VIP KO mice exposed to long and short photoperiods are indistinguishable from those exhibited by wild type mice (**Chapter 4, Fig. 3 and Fig. 4**). This implies that SCN neurons of VIP KO are still able to adapt the phase distribution to the day length while under light-dark conditions.[1] However, these properties are not maintained when environmental light input is removed, reflecting a severe deficit in endogenous seasonal encoding in the absence of VIP. Inability to adapt to seasons is problematic, as it may result in catabolic states at inappropriate seasonal times of food scarcity, when energy saving anabolic states would have been more advantageous to survival, for example.

VIP is also expressed in many tissues outside the SCN and the brain, including the gut, pancreas, and cardiopulmonary system. VIP KO mice therefore also exhibit other abnormalities, like altered immune responses[2,3] and spontaneous cardiomyopathy.[4] Although we cannot exclude the possibility of these factors influencing our results, it is likely that the loss of memory for day length encoding is mainly explained by the lack of VIP in the SCN specifically.

To our knowledge an absence of VIP has not been reported in humans, but several pathological conditions have been associated with upregulated VIP levels. VIPoma is a rare tumor with VIP overproduction by pancreatic non-beta islet cells and a number of human cancers overexpress VIP receptors.[5] Mice with overexpression of VIP receptors in the SCN adapt quicker to phase shifts and have a shorter period in constant darkness.[6] It is unknown whether patients with VIPoma exhibit circadian disturbances. While the absence of VIP specifically might not be directly translational to humans, the examination of circadian properties in VIP KO mice demonstrates basic functionalities of the circadian system.

Appropriate input to the circadian system can compensate for deficient individual components and enhance rhythmicity

Although VIP is needed for seasonal encoding, the studies in **Chapter 4** also demonstrate that SCN function in light-dark conditions is relatively well maintained in the absence of VIP. On the other hand, many peripheral rhythms are disrupted in mice lacking VIP or its receptor, including rhythms of adrenocorticotropic hormone, cortisone, heart rate, behavior, and sleep-wake patterns.[7-12] *In vitro* examination of the SCN also revealed disruption of circadian rhythmicity[13,14] and weakened synchrony amongst SCN neurons in VIP KO mice.[15,16] Additionally, rhythmic molecular expression of clock genes in their SCN is attenuated.[17-19] Nevertheless, VIP KO mice display MUA rhythms of remarkable quality under LD12:12 conditions (**Chapter 4, Fig. 1**). Thus, the explanation for the disrupted rhythms in the periphery in VIP KO mice must lay in the output pathways of the SCN. As VIP function is absent throughout the body in VIP KO mice and many SCN projections to downstream targets are VIPergic,[20] the signal may be disrupted at this level. A similar phenomenon has been described in Per KO mice: the SCN rhythm of these mice is attenuated or abolished *in vitro*, yet their rhythm amplitude *in vivo* is identical to wild type mice.[21]

The fact that MUA rhythmicity is so well maintained in VIP KO mice at the neuronal level *in vivo* while VIP is so important for neuronal coupling demonstrates that compensation takes place and that there are other synchronizing factors. SCN cells secrete more than 100 peptides, some of which with potential coupling capacities, like GABA, GRP and AVP.[22] Light directly stimulates SCN firing rate through glutamate[23] and the neuropeptide pituitary adenylate cyclase activating peptide (PACAP).[24-26] The neuronal light response in the ventral

SCN,[27] as well as the behavioral response to light,[10] is unaffected by the loss of VIP. In VIP KO mice, light still suppresses behavioral activity, while darkness enhances active behavior.[10] The obvious lack of light input in *in vitro* experiments may account for the severe effect of VIP loss in these experiments *vs.* the sustained rhythmicity under light-dark conditions in *in vivo* experiments. Furthermore, *in vitro* experiments do not detect the influence of other brain areas which have been shown to directly regulate SCN.[28] Thus, the preserved rhythms in VIP KO mice may be explained by the direct effect of light or feedback from other brain areas. This is supported by evidence that rhythmicity in VIP KO mice is reinforced by providing timed access to a running wheel.[29,30]

Timing of behavioral activity strongly reflects SCN rhythmicity

In wild type mice, appropriate timing of behavioral activity also has the potential to enhance circadian rhythms (**Chapter 6**). Using simultaneous *in vivo* behavioral and neuronal MUA recordings in freely moving mice, we observed that MUA was acutely suppressed during short the episodes that the animal was active (**Chapter 6, Fig. 1**). Thus, behavior at night enhances the trough in MUA that occurs at night, while daytime rest allows for a maximal peak in MUA. While less intensive behaviors such as grooming also associate with a suppression in neuronal activity, more intense types of behavior like walking were associated with a deeper suppression of neuronal activity (**Chapter 6, Fig. 4**). The duration of activity did not influence suppressions, as episodes of ultra-short behavioral activity associated with suppressions of the same magnitude as longer behavioral episodes. Since mice are nocturnal animals and activity-associated suppression is superimposed on the circadian rhythm, behavior at the proper time can boost clock amplitude (**Chapter 6, Fig. 6**).

The circadian rhythm of behavioral activity is strongly correlated with the amount of rhythmicity of the SCN. For instance, constant light (LL) decreases the strength of the behavioral as well as the neuronal rhythm (**Chapter 8, Fig. 1, Fig. 2**). LL decreases rhythmicity more in some than in other mice and the effect of LL varies over time, generally with rhythm disruptions becoming progressively outspoken with a longer duration of LL exposure. Interestingly, in mice recordings that displayed neuronal rhythms of higher amplitude, concomitant behavior also followed a more robust and circadian pattern (**Chapter 8, Fig. 2**). The association between time-scale behavior and acute neuronal suppressions remains present in LL, as well as in constant darkness (DD) (**Chapter 6**). Furthermore, the half-maximum downward and upward slopes of the MUA rhythm act as thresholds that determine the onset and offset of behavioral activity.[31] These thresholds are maintained in different photoperiods and in non-24 hr *t*-cycles. The downward slope of MUA especially was strongly correlated with the onset of behavior, which is consistent with the observation that behavioral onsets on nocturnal rodents are more precise and acute compared to their offsets.[32]

It has additionally been shown that mice fed a high fat diet display attenuated rhythmicity in behavior and that placing a running wheel in their cage, allowing them to exercise, enhanced circadian rhythmicity in behavior.[33] The presence of a running wheel also enhanced circadian amplitude of clock neurons *in vitro* in aged mice.[34] Thus, exercise appears to "rescue" rhythmicity in animals with weakened rhythms. Regular wild type mice probably also benefit from exercise, as giving access to a running wheel enhanced circadian rhythmicity of body temperature and cortisone levels,[35] as well as the clock gene rhythm in several peripheral organs. In addition, exercise directly influences the central clock, as it enhanced the amplitude of the SCN neuronal activity rhythm *in vivo.*[36]

It was difficult to distinguish cause and effect in our recordings, as we recorded on a 1-second bin timescale and behavioral activity and neuronal suppression appeared to occur simultaneously. We hypothesize that behavior causes the suppression in neuronal firing, because the MUA signal remains suppressed after termination of behavioral activity. Furthermore, behavior elicited by an external stimulus had a direct effect on neuronal activity (**Chapter 6, Fig. 5**). Additionally, *in vitro* recordings of SCN slices do not exhibit suppression-like patterns in neuronal activity rhythms. *Vice versa*, artificial suppression of MUA by infusion of tetrodotoxin (TTX) during the resting phase elicits behavioral activity, probably because TTX silences GABA-ergic SCN neurons that normally inhibit behavioral activity, demonstrating that SCN activity levels are also a causal factor for initiating behavior.[31] Together, these results show a bidirectional relationship between the SCN and behavioral activity.

Differential effects of spontaneous vs. induced behavior on neuronal firing

Whereas spontaneous behavior is associated with a reduction in neuronal activity, increases in MUA are observed with behavior that is induced by noise production or by small movements of the cage floor (**Chapter 6, Fig. 5**). The kinetics of the neuronal response to induced behavior shows clear differences to that induced by spontaneous activity. At the start of the behavior, neuronal firing is acutely diminished and remains at a low level throughout both types of behavioral activity. After termination of induced behavior, neuronal firing levels climb back up more slowly to their original levels in an exponential function in comparison to spontaneous behavior (**Chapter 6, Fig. 2, Fig. 5**).[37] These findings may be caused by usage of a different subset of neurons or neurotransmitters in either situation. In our experiment, mice froze upon disturbance, an activity that is associated by type 2 theta activity in the hippocampus that uses acetylcholine.[38] As cholinergic neurons innervate the SCN from forebrain and brainstem cells, it is well possible that this system mediates the augmentation in SCN activity.[39] It is unlikely that the increase in SCN activity arises from the activation of a stress response, since it would require more time, hamsters

produce little cortisol after behavior induction[40] and the SCN lacks cortisone receptors.[41,42] Thus, the involvement of the arousal or anxiety system in the induced-behavioral SCN excitatory response is more likely.

The relatively gradual exponential return to baseline levels implies the usage of a slow neurotransmitter system, such as hypocretin or another neuromodulator. *In vitro* application of hypocretin demonstrates alteration of SCN activity with a similar characteristic slow recovery[43] and behavioral activity leads to increased *c*-Fos expression in hypocretin cells in the lateral hypothalamus.[44]

While it would be beneficial for an organism to display most spontaneous behavior during the active phase and rest during the inactive phase, induced behavior may be elicited by a stimulus that requires active behavior regardless of the time of day. For example, an alert state is essential in response to acute danger, so while an increase in SCN activity may simply be a byproduct of activation of the arousal or anxiety system, it may also help to signal the body to be active and alert whenever this is required for an external stimulus.

Photic phase shifting capacity is influenced by behavioral aspects and caffeine

The circadian system must be responsive to phase shifts, in order to adapt to a new environment, but it should not be disrupted too easily to provide temporal stability. Behavior, light and sleep deprivation are examples of Zeitgebers that can induce phase shifting. When applied at the beginning of the subjective night (CT14), phase delay to a light pulse is of maximal magnitude and the behavioral rhythm shifts by about 2 hrs in one day (**Chapter 5, Fig. 1**).[45] The nonphotic phase response curve follows a different pattern and its shape is similar for novelty-induced wheel running and sleep deprivation.[46,47] Sleep deprivation at CT14 induces a phase-delay of about 45 minutes (**Chapter 5, Fig. 1**). Photic and nonphotic effects are not additive, as a light pulse combined with sleep deprivation at CT14 resulted in a phase shift of about an hour (**Chapter 5, Fig. 1**).[48,49] Behavioral activity also influences phase shifting magnitude. For example, phase shifts are attenuated when the running wheel is blocked, so active behavior accelerates re-entrainment to shifted environments.[34,50,51] In studies described in **Chapter 5**, we show that the light response of SCN neurons is attenuated in sleep deprived mice, which may reflect the reduced photic phase-shifting capacity (**Chapter 5, Fig. 2**).

Mechanistically, brain adenosine levels rise in the absence of sleep and fall upon initiation of sleep. **Chapter 5** provides evidence for a role of adenosine in the regulation of the light response of the SCN and the restorative effect of caffeine on the amplitude of light responses in the sleep deprived state.

In modern society, sleepiness is often commonly counteracted by the usage of caffeine, an adenosine receptor antagonist. Mice given sleep deprivation and caffeine simultaneously displayed light responses that were indistinguishable from well rested mice given light pulses. Exposure to LL input lengthens endogenous period (**Chapter 8, Fig. 1, Chapter 5, Fig. 4**) and the administration of caffeine during LL exposure leads to an extreme lengthening of the period (**Chapter 5, Fig. 4**). Mice consuming caffeine in DD also lengthen their period, but to a much lesser extend (**Chapter 5, Fig. 4**). These observations support the hypothesis that caffeine enhances light sensitivity of the circadian system. Caffeine also exerts a direct effect on SCN function, as it lengthened the endogenous period in the absence of light input (**Chapter 5, Fig. 4**) and application of caffeine induces a phase delay of peripheral clocks.[52]

Besides adenosine effects, the loss of sleep results in increased SCN serotonergic activity, reduced SCN electrical activity,[28] and metabolic changes (**Chapter 2, Chapter 3**). Behavior increases 5-HT and NPY levels in the SCN[53,54] and GABA-A mimics non-photic shifting of SCN phase.[55] Therefore, these neurotransmitters are additional candidates that can mediate the effect of sleep loss on the SCN.

In acutely sleep-deprived humans, phase advances are attenuated in response to bright light.[56] The attenuation was modest, but small discrepancies between internal and external phase may already significantly affect well-being.[57] Chronic sleep deprivation in humans affects light-induced phase shifting magnitude more severely.[58,59] Thus, in humans that are sleep deprived, jet lag following a trans meridian flight may be worse. Furthermore, adaptation to altering timings of behavior that may occur in shift work or in social jet lag may be more difficult in sleep-deprived individuals. In humans, caffeine suppresses the increased nocturnal release of melatonin and the evening drop in body temperature, especially when combined with light exposure.[60] Therefore, intake of caffeine may also alleviate the sleep loss-induced effects through restoring sensitivity to light in humans.

Characteristics of altered circadian rhythms induced by SCN lesions, LL or chronotype differences

Healthy circadian rhythms display a high amplitude difference between day and night and are timed in synchrony with the environmental light-dark cycle. There are several states or behaviors that affect circadian amplitude, timing or both. In studies described in **Chapter 7**, SCN lesions were performed in mice which completely abolished behavioral circadian rhythmicity (**Chapter 7, Fig. 1, Fig. 2**). Destruction of the central pacemaker results in desynchrony between peripheral rhythms, but not necessarily in elimination of tissue rhythmicity. Certain tissues in SCN-lesioned and behaviorally arrhythmic mice still display oscillations, although their amplitude is decreased.[61] Thus, by using this model, the role of the central clock can be elucidated.

A less drastic method, used in **Chapter 8,** that also affects the SCN is exposure to constant light, which removes circadian environmental cues and decreases the amplitude of circadian rhythms. The diminished rhythmicity in the SCN in LL (**Chapter 8, Fig. 2**)[62,63] is caused by a desynchrony amongst SCN neurons.[64] The weak remaining rhythms in LL may be generated by a subpopulation of synchronous cells. As a consequence, rhythms in peripheral tissues[65] and in behavior (**Chapter 8, Fig. 1**)[62] are also attenuated. The amplitude reduction of locomotor rhythms in LL appears to be mediated by VIP, since LL does not affect behavioral rhythmicity in VIP KO mice.[66] The benefit of the usage of LL exposure *vs.* SCN lesion models is that effects are reversible when the environmental rhythm is restored. Interestingly, circadian rhythm parameters of mice recover immediately upon the first dark exposure after long term LL (**Chapter 8, Fig. 1**)[67], implying that their phase is reset instantly. Using this protocol, not only the SCN but also other light-recipient brain areas are registering light input, including the intergeniculate leaflet, the visual cortex, and the olivary pretectal nucleus responsible for the pupillary light reflex.[68] Therefore, effects may be partly mediated by parts of the brain outside the circadian system. Thus, the LL protocol successfully mimics the aberrant light input that characterizes modern society, by activating equivalent SCN and extra-SCN light-induced pathways.

In **Chapter 9**, we compared characteristics of Morning and Evening chronotype's. Due to external demands, each chronotype may be forced to be active at "wrong" internal times. For example, Evening type's may be required to get up early to go to work, whereas Morning type's may be "forced" to stay out late for social gatherings while their internal clock dictates sleep. The phase relationship between peripheral clocks, environment[69] and behavior[70,71] is reported to be chronotype-dependent. The circadian amplitude of behavior likely does not depend on chronotype.[72]

Therefore, the circadian consequences of SCN lesions and LL are mainly a reduction in amplitude whereas chronotype mainly causes altered timing of certain behaviors in relationship to the environment and the internal rhythm.

Contribution of altered sleep in SCN lesions, LL, and chronotype on health effects

The control of the circadian rhythm of sleep lays in the SCN. Therefore, rhythmicity in sleep is absent in SCN-lesioned animals[73,74] and it is diminished in LL.[75] The SCN may regulate additional aspects of sleep beyond timing, such as sleep duration and homeostasis. Besides abolishment of rhythmicity, SCN lesioning also lengthened total sleep time in squirrel monkeys[73]and in mice,[74] due to an increase in time spent in the lighter stages of NREM sleep.[73,74] Furthermore, the compensatory increase in NREM sleep after sleep deprivation is absent in SCN-lesioned mice.[74] Rats exposed to LL spent longer in NREM at the expense of

time awake[75,76] and time spent in REM sleep.[75] Thus, effects of LL and SCN lesions on sleep go in a similar direction. For interpretation of results presented in **Chapters 7** and **Chapter 8**, sleep alterations may have exerted minor effects on outcomes, although sleep is certainly not shorter.

Chronotype determines the mid-sleep time (MST), the clock time when half of the total sleep duration is completed, with Evening types having a later MST compared to Morning types (**Chapter 9, Fig. 1**).[77] Without social constraints, both chronotypes exhibit sleep of similar duration and quality.[78,79] However, in everyday life, Evening types generally accumulate sleep debt over the work week that they replenish by extending sleep on weekends.[72,77] Obese, short sleeping Morning and Evening types do not differ in sleep duration on work- or weekend days, overall sleep quality or daytime sleepiness (**Chapter 9, Table 2**). However, selection for short sleep places both chronotypes on the short-sleeping extreme of the normal distribution of sleep duration, increasing their likelihood to experience worsened sleep quality and excessive daytime sleepiness and thereby differences in sleep parameters between chronotypes may become obscured. Interestingly, obese short sleeping Evening types overestimate their sleep duration on workdays, indicated by a discrepancy between self-reported sleep diary and objectively measured actigraphy measurements (**Chapter 9, Table 2**). The fact that these Evening types appear to be less conscious of their sleep debt may render them less prone to ameliorate their sleep length. The distribution of the chronotype score in a population that was selected based on obesity and short sleep is slightly skewed towards eveningness in comparison to the normal population (**Chapter 9**),[79,80] indicating that the Evening chronotype is somewhat more likely to be obese and short-sleeping. The separate contribution of obesity and short sleep in skewing scores towards eveningness cannot be distinguished. Thus, the effects of chronotype on metabolism presented in **Chapter 9** are caused by differences in sleep and behavioral *timing* relative to the environment, but only apply to a short-sleeping obese population.

Lesions of the central clock in the SCN lead to metabolic alterations

SCN-lesioned mice lack the physiological nocturnal increase in oxygen consumption, energy intake, behavioral activity, and respiratory ratio (**Chapter 7, Fig. 3**). Their total oxygen consumption and behavioral activity levels are similar to sham-lesioned mice, while total energy intake is reduced by 26%. Despite their lower food intake, SCN-lesioned mice are 17% heavier, due to an increased fat mass (**Chapter 7, Fig. 2**). The respiratory ratio is lower during the day and night in SCN-lesioned mice, so the response to food intake is directed more toward fat oxidation instead of carbohydrates. The inability to upregulate carbohydrate oxidation appears to be caused by a deficient tissue glucose uptake due to insulin resistance and has been associated with impaired insulin sensitivity in humans.[81]

Hyperinsulinemic-euglycemic clamp studies confirm increased insulin resistance in SCN-lesioned mice, since they require lower glucose doses to maintain euglycemia (**Chapter 7, Fig. 4**). Endogenous glucose production is suppressed about 12 times less effectively (7% *vs*. 84%) during the hyperinsulinemic period in SCN-lesioned *vs.* SCN-intact mice, indicating severe hepatic insulin resistance, while glucose uptake by peripheral tissues is not affected by SCN-lesioning (**Chapter 7, Fig. 5**). Thus, while total levels of metabolic measures are similar, SCN-lesions induce obesity and severe hepatic insulin resistance. Therefore, these metabolic defects appear to be caused by either the lack of rhythmicity in SCN-lesioned animals or due to a shift in homeostatic set point. For instance, the mild weight gain in SCN-lesioned mice may be caused by reduced brown adipose tissue activity, because the SCN exerts an excitatory influence on the VMH neurons involved in activating brown adipose tissue.[82] Possibly, SCN lesions remove the physiological inhibitory SCN input to the adipose tissue, thereby increasing the basal rate of lipolysis. Indeed, levels of free fatty acid are increased in SCN-lesioned mice (**Chapter 7**, **Table 1**), which is an additional risk factor for developing insulin resistance.[83]

By abolishing rhythmicity due to SCN lesioning, the timing of food intake and of energy expenditure may not be optimally adjusted to one another. Physiological circadian rhythmicity in hormones regulating metabolism (**Chapter 2**), such as leptin, is absent in mice with SCN-lesions.[86] Additionally, neither basal glucose and insulin levels nor glucose and insulin responses to meals[87]and glucose utilization inhibitors[88] display a circadian variation in SCN-lesioned animals. An imbalance between energy intake and expenditure is detrimental for glucose homeostasis. The importance of well-timed eating rhythms is supported by studies in rats: these nocturnal animals gain more weight when fed only during the day.[89-91] These protocols are different from SCN lesioning, because these animals eat “against their clock” instead of having an absent central clock. A similar phenomenon is observed in mice exposed to LL (**Chapter 8**).[62,92] LL quickly induces a mildly increased weight that remains stable over time and increased unfasted glucose levels at CT0 (**Chapter 8, Fig. 1**). When environmental rhythmicity is restored in mice previously exposed to long term LL, weight and glucose levels quickly normalize (**Chapter 8, Fig. 1**). These findings imply that the mechanisms responsible for the LL-induced weight increase are fast-acting and induce stable changes.

Damage to the PVN and VMH increases metabolic alterations in SCN-lesioned mice

The SCN partly controls hepatic glucose homeostasis through time-dependent GABA-ergic and glutaminergic cross-communication through the PVN.[93] Mice with SCN lesions extending to the PVN and VMH display a significantly more severe phenotype of obesity and

insulin resistance compared to mice with exclusive SCN-lesions (**Chapter 7, Fig. 6**). Unlike mice with selective SCN lesions, mice with extended lesions also exhibit peripheral insulin resistance, probably resulting from their increased fat mass. Unilateral damage to the PVN does not additionally affect weight in SCN-lesioned mice (**Chapter 7, Fig. 6**), suggesting the PVN needs to be bilaterally damaged in order to influence weight. The proximity of the anatomical location of SCN and surrounding metabolic regulatory areas underlines the importance of careful positioning of hypothalamic site-specific lesions and subsequent histological verification.

Diminished circadian rhythm amplitude induces a frail state

Studies in **Chapter 8** describe that mice exposed to LL display a diminished muscle function, more brittle bones, and an altered function of the immune system, all contributors to a state of frailty.[94] Short term exposure to a LL regime impacts muscle strength and endurance and these parameters remain affected in the long run. Long term LL exposure also induces worsening of trabecular bone parameters. After 24 wks of LL, the amount of trabecular bone volume and thickness are diminished and the shape of trabeculae is more rod-like, which makes the bone more brittle and easier to break. Effects on the immune system change over time of LL exposure. Two wks of LL induce some changes in the responsivity of the immune system. Eight wks in LL results in a pro-inflammatory state, characterised by higher circulating levels of neutrophils and increased pro-inflammatory cytokine responses to an immune challenge. With a longer duration in LL, immune homeostasis and responsivity normalize, suggesting adaptation of the immune system to the state of LL.

Many processes involved in bone restructuring, muscle metabolism and immune function display circadian rhythms.[95-97] We did not find evidence for structural changes in the muscles or in its metabolism at a single time point, nor for differences in motivation to perform the functional muscle tests, and nor for mediation by weight or overall behavioral activity for the worsened muscle function in LL. Therefore, we propose that the deterioration in muscle function, bone microstructure and immune function results from an LL-induced disruption of circadian processes important for these functions.

Constant light as a model for an aged clock

LL diminishes the amplitude of the central clock in the SCN in a similar way to aging. As LL exposure may mimic the effects of an old clock, exposure of young animals to LL may unravel the specific contribution of the old clock to certain age-related conditions. The question is how comparable the circadian effects of LL and aging are and whether these effects persist across species. In LL and in aging, the development of the circadian system has been normal.

Upon LL exposure in mice[64,67] and during the aging process in mice[98] and in humans,[99] the amplitude in behavioral rhythmicity progressively weakens (**Table 1**). Likewise, rhythmicity of several peripheral rhythms is attenuated in all three conditions (**Table 1**).[67,100-103] The SCN degenerates and loses VIP neurons in the process of human[104] and murine[105] aging. Short term LL does not affect SCN volume, but reduces the number of glial cells in the circadian system.[106] More importantly, the rhythmicity of the SCN neuronal and clock genes is diminished *in vivo* in LL-exposed[62,64] and in aged[100,107] mice (**Table 1**). *In vitro* experiments indicate that the reduction in amplitude could be attributed to increased desynchronization between individual neurons in these conditions.[64,98,107] Human postmortem studies showed a decreased rhythm in vasopressin cell number in aged subjects (**Table 1**).[104]

There have not been many studies on constant light exposure in humans, but it is likely that circadian effects would be similar compared to mice, because of the resembling responsivity of their circadian systems to light. SCN activity in humans (measured by blood oxygen levels on fMRI)[108] and in mice (measured by electrophysiological recordings)[26] is most responsive to light at night. Fittingly, the human and murine photic phase response curves are very similar.[109,110] Furthermore, SCN neuronal firing levels are high during the day and low during the night in both diurnal[111] and nocturnal animals (**Chapter 4, Chapter 5, Chapter 6, Chapter 8**).[26] Bright light exposure at night significantly attenuated peripheral rhythmicity in humans,[112] similar to the diminished amplitude in behavior observed in LL-exposed mice presented in **Chapter 8**.

The period in behavior is longer in LL-exposed and in aged mice,[64,98] while it is shorter in aged primates[113] and in fibroblasts of elderly subjects.[114] Thus, LL and aging exert similar effects on period within the mouse species, but effects differ between species. Similarly, the total duration of NREM sleep is increased in LL[75,76] and in aging[98] in rodents, while elderly humans appear to sleep less and display a decrease in sleep efficiency (**Table 1**).[115] In rodents, sleep in both conditions is more spread throughout the 24h cycle, while elderly humans attempt to condensate most sleep during the night, which may explain their shorter sleep duration. However , elderly humans demonstrate more resilience to sleep deprivation,[115] so aging-effects on sleep may truly differ between species.

Generally, mice exposed to short term LL (**Chapter 8**),[62] and old mice[98,107] are less active. Comparably, aged humans display less active behavior (**Table 1**).[99]

Phase shifting capacity in aged mice and humans is altered in aging,[116-119] both depicting a slower adaptation to phase advancing light-dark cycles (**Table 1**). The examination of phase shifting by light pulses is not possible in LL, hindering a comparison between LL and aging in mice. However, we can conclude that effects of aging on phase shifting capacity are similar across mice and human species.

Thus, in all three conditions (LL and aging in mice, and aging in human) the circadian rhythm amplitude is diminished, likely mainly due to a similar mechanism consisting of

desynchronization of individual neurons. Likewise, behavioral activity is diminished in all conditions. LL and aging also exert comparable effects on period and sleep in mice, but aging effects these parameters differently in humans. Thus, there are some differences between species in age-induced effects on period and sleep, but the resemblance of circadian parameters between aging and LL in the mouse is remarkable and provides an opportunity for examining effects of an "old clock" in otherwise young animals.

Table 1: Comparison of circadian parameters in constant light and in aging in mice (unless other animals are mentioned specifically), and translation to aged humans.

	LL (mice)	Aging (mice)	Aging (humans)
Circadian rhythm amplitude:			
Behavior	decreased (**Chapter 8**)[64,67]	decreased[98]	decreased [99]
SCN	decreased MUA (**Chapter 8**)[62] and mPer1/2 *in vivo* [67]; desynchronization Per1::GFP expression *in vitro*[64]	decreased MUA *in vivo*[107] and *in vitro*[98], Clock/Bmal1 *in vivo*[100] and Per2::luc *in vitro*[107]; desynchronization neuronal firing *in vitro*[98]	decreased (vasopressin post-mortem)[104]
SCN outputs	decreased mPer1/2 in liver[67]	decreased Clock/Bmal1 in brain,[100] Per1/2/3/Bmal1/Cry2 in liver and heart [101]	decreased melatonin[102] and body temperature[103]
Other parameters			
Period	longer (behavior)[64]	longer (behavior)[98]	shorter (fibroblasts) [114] or similar (behavior by forced desynchrony)[216]
Phase shifting (light pulses)	not possible	slower advance (behavior and peripheral rhythms tissues)[116]	decreased advance [117,118]; similar delay [117,119]
Sleep	increased sleep time, more NREM, less REM (rats)[75]	increased sleep time, more NREM, less REM[98]	decreased sleep time, less efficiency[115]
Activity	less[62]	less[98,107]	less[99]

Association between circadian rhythms and metabolic alterations in humans

In humans, disrupted circadian rhythms have been linked to metabolic alterations. Shift workers are at increased risk for developing obesity and type 2 diabetes. This has been reported consistently in large cross-sectional[120] and prospective studies,[121,122] in men[121]and in women.[120,122] The effect appears to be dose-dependent, meaning that the odds for metabolic disorders appears to increase with the duration of shift work.[122] Additionally, shift work is associated with an increased incidence of breast cancer,[123] osteoporosis,[124] and bone fractures.[125]

There also is evidence for a link between genetic variation in clock function and health outcomes. Allelic variation in circadian related genes *Clock*[126,127] and *Rev-erb alpha*[128] has been linked with obesity. Certain polymorphisms of the clock genes *Per2,*[129] *Bmal1,*[130]

Cry2,[131] and *MTNR1B*[132-134] relate to higher fasting blood glucose levels and increased risk for type 2 diabetes. Furthermore, *Clock,*[136,137] *Cry2,*[137] and *MTNR1B*[137] gene variants determine the magnitude of improvement of metabolic parameters in response to certain diets. *Per1* polymorphisms have been associated with the risk for osteoporosis in postmenopausal Korean women[138] and it has been proposed that the relationship between night shift work and breast cancer may be mediated trough clock gene polymorphisms.[139]

These studies are strictly correlational, but experimental human studies reveal a causative role of circadian disruption in metabolic alterations. For instance, short term circadian misalignment induces metabolic disturbances,[140] including a decreased glucose tolerance.[141] When combined with sleep restriction, resting metabolic rate decreases and postprandial glucose levels are elevated, putting individuals at risk for obesity and diabetes.[142]

Timing of behavior affects health parameters

A chronotype score progressing towards eveningness associates with unfavorable metabolic parameters, indicating that behavioral timing affects health. Lean Evening types tend to have an increased BMI,[143] a higher resting heart rate[144,145] and an altered heart rate variability.[145] In studies in **Chapter 9**, the association between chronotype and metabolic parameters is examined in short sleeping obese individuals. In these individuals, chronotype scores towards eveningness also relate to increased BMI and neck circumference, while relating to lower circulating levels of HDL (**Chapter 9, Fig. 3**). Additionally, Evening types in this cohort have higher values of ACTH, 24h urinary levels of norepinephrine and epinephrine and a higher resting heartbeat (**Chapter 9, Fig. 4, Table 4**), indicating an activation of the sympathoadrenal system. HDL, ACTH and resting heartbeat were only measured in the morning, so differences could have resulted from differences in phasing of circadian rhythms. However, also 24h levels of sympathetic values and anthropometric characteristics differed.

These findings may have been caused by altered phase relationship in behavioral timing *vs.* internal processes or differences in behavior *per se*. Short sleeping obese Evening types have a later sleep timing and a later food intake. Overall, they eat less often and tend to eat larger portions (**Chapter 9, Fig. 2, Table 3**). These altered patterns[146-148] and timing[149,150] of food intake have been associated independently with a higher BMI and increased risk of weight gain. Additionally, meal composition depends on the time of day, as meals consumed after 20:00 contain less protein (**Chapter 9**). As protein is a more satiating compound compared to carbohydrates, meals consumed after 20:00 may therefore be less successful in reducing appetite.

Combined effect of short sleep and obesity on neurocognitive function

In **Chapter 10**, we describe studies examining the neurocognitive deficits in a population of short sleeping obese individuals. As expected, many of these individuals display neurocognitive deficits: the mean score on the GDS is 41.7, which is below average, and 44% scores in the impaired range. In comparison, 10% of the normal population has impaired GDS scores.[151] Executive function is most often impaired (51%), followed by motor skills (42%), attention (36%), and memory (33%) (**Chapter 10, Fig. 1**). Obese subjects are already impaired in executive function[152-157] and since short sleeping individuals lack the protective effect of a good night's sleep on memory and attention,[158] effects on executive function may be more profound in short sleeping obese subjects. *Vice versa*, executive dysfunction may result in further deteriorated performance on more complex memory and attention tasks.

Worse sleep quality and sleep efficiency contribute to a further deterioration in GDS in short sleeping obese individuals (**Chapter 10, Table 4**). Thus, even though all participants sleep short, a decreased sleep quality can still aggravate cognitive function. The absence of an effect of anthropometric parameters may result from a ceiling effect of obesity. Thus, obesity *per se* may influence neurocognitive function, but since all individuals were obese, additional obesity does not further exacerbate neurocognitive deficits. It is hard to determine the exact cut-off value for this potential ceiling effect.

Deficient neurocognitive functioning is associated with lower levels of circulating dopamine, urinary norepinephrine and free cortisol in short sleeping obese individuals (**Chapter 10, Table 1, Table 4**). Levels of these substances are only lower in individuals deficient in memory- and attention-related neurocognitive tasks, while a deficient motor or executive function did not affect levels. Dopamine, norepinephrine and cortisol are substances that are widely distributed throughout brain areas involved in neurocognitive function. The major site of memory processing and cognitive control, the prefrontal cortex, is highly sensitive to dopaminergic input.[159] Norepinephrine, a neurotransmitter that is synthesized from dopamine,[160] also acts in the prefrontal cortex[161] as well as the hippocampus,[162] where it facilitates long-term potentiation a process important in memory formation. Although peripheral levels of catecholamines and cortisol do not determine the regional distributions of these levels within the brain and therefore do not necessarily reflect central neurotransmission and intracellular processes,[163] there is crosstalk between the central and peripheral systems.[164] In several psychiatric disorders, such as anxiety and depression, central and peripheral systems are affected[164] and the administration of medications such as levodopa and antidepressents influences plasma, urinary, and central levels.[164,165] High circulating levels of cortisol have been associated with hippocampal atrophy, demonstrating a central effect of peripheral concentrations.[166-168]

Results described in **Chapter 10** are consistent with existing literature. In monkeys, depletion of dopamine resulted in worse performance on working memory tasks[169,170] and

dopamine receptor agonists increased working memory of young human volunteers, while not affecting motor function[171-176] The first site affected in Alzheimer is the locus coeruleus, the main source of norepinephrine.[177] Moreover, application of norepinephrine appears to reverse AD pathology.[177] Disturbances in cortisol release have been described in cognitive impairment, but results have been variable.[178-180] Although our study described in **Chapter 10** is strictly correlational, the experimental studies on norepinephrine[177] and dopamine[169-176] mentioned above advocate a causative role of substance levels in decreased cognitive functioning. However, it should be noted that peripheral catecholamine and cortisol concentrations do not always predict central levels accurately. This especially holds for the catecholamines since the brain contains its own catecholamine producing systems[163-168]

Sleep deprivation and obesity also affect dopamine, urinary norepinephrine and free cortisol levels (**Chapter 2**). Thus, neurocognitive deficits observed in our short sleeping obese cohort may be mediated through changes in the levels of these substances. Alternatively, short sleep, obesity, and neurocognitive function may share common etiologies due to altered dopamine, norepinephrine or cortisol systems. For example, as food intake stimulates dopamine release in the brain reward circuit and in the circulation, dysfunction of the dopaminergic system may lead to excessive reward seeking behavior, impaired executive function and ultimately obesity.[181,182] In addition, dopaminergic transmission is needed for the occurrence of REM sleep and depletion of REM sleep results in neurocognitive deficits.[183] Thus, all processes likely interact in a bidirectional manner.

Association of sleep with musculoskeletal parameters

Sleep is also associated with musculoskeletal health, as decribes in the study in **Chapter 11**. In this study, 916 individuals of the Netherlands Epidemiology of Obesity (NEO) study were enrolled. Participants were 45-65 yrs old, generally slept relatively well (7 hrs per night, 85% reported sleeping fairly or very good) and averagely had a low incidence of osteopenia in the spine or femoral neck (37%) and sarcopenia (19%). We observed that decreased sleep quality and later sleep timing associates with an increased risk for osteopenia and sarcopenia in models that were adjusted for confounders (**Chapter 11, Fig. 1**), consistent with most previous studies.[183-187] Although this was a cross-sectional study and therefore causality cannot be determined, we hypothesize that sleep exerts an effect on osteopenia/ sarcopenia and less so *vice versa*. The deepest sleep stages may be reached less often by individuals experiencing declined sleep quality,[188,189] while these are the times of growth hormone secretion[190] and are considered most “restorative”. Experimentally fragmenting sleep resulted in higher cortisol and catecholamine levels, which may negatively affect the musculoskeletal system. A later sleep time may affect the musculoskeletal system through comparable mechanisms, as the evening chronotype has been associated with higher levels

of catecholamines (**Chapter 9, Fig. 4**). Effects may also be explained by a more unhealthy lifestyle that is associated with later bed times (**Chapter 9, Fig. 2**), although we adjusted analysis for smoking, alcohol intake and whole body fat mass and results remained significant. Interestingly, sleep duration did not associate with musculoskeletal parameters, consistent with a previous study in Caucasians.[191] Our participants slept relatively well, and more severe sleep deprivation may be necessary to affect musculoskeletal health. Alternatively, sleep quality and timing are more important factors in musculoskeletal health than sleep duration. The associations between sleep parameters and osteopenia/sarcopenia were independent from muscle mass/BMD, respectively, indicating that these associations exist in parallel but are not influenced by one another.

Associations between sleep parameters and musculoskeletal health were stronger in women than in men, which may be due to differences in sleep and musculoskeletal health *per se* and may be influenced by sex hormones and behavioral differences (**Chapter 11, Fig. 2**). Small differences in the effect sizes of the associations per menopausal status were present (**Chapter 11, Suppl. Fig. 1**). In postmenopausal women, osteopenia associated somewhat weaker with sleep quality and timing while sarcopenia displayed a stronger association compared to pre- and perimenopausal women, which could be due mediated by differences in estrogen levels.

Recovery of functions by strong rhythms and improvement of sleep

The study presented in **Chapter 8** describes that LL exposure negatively effects muscle function and bone microstructure. These adverse effects are alleviated by the reintroduction of a light-dark regime. The neuronal rhythm in the SCN recovers immediately upon first dark exposure after long term LL as well as the rhythm strength of the behavioral rhythm (**Chapter 8, Fig. 1, Fig. 2**). At the first time point after LD12:12 exposure (t = 2 wks), muscle function of mice previously kept in LL is already indistinguishable from mice that were on LD12:12 throughout the experiment (**Chapter 8, Fig. 3**). Similarly, while all trabecular parameters indicate a more brittle bone structure at 24 wks of LL exposure, no differences in bone structure are observed at 8 wks after recovery of the light regime (**Chapter 8, Fig. 4, Suppl. Fig. 3**). Therefore, recovery of environmental rhythmicity in light exposure has a fast-acting beneficial effect on health.

An advantageous effect of exposure to strong environmental lighting cycles has been demonstrated in humans. Enhancing the diurnal variability of lighting in nursing homes improved sleep, attenuated cognitive deterioration, and increased social and physically active behavior in residents.[192,193] Likewise, preterm infants at the neonatal intensive care unit gained more weight when robust light-dark cycles were installed.[194,195] Exposure to solely natural light, either by mimicking pre-historic living conditions[196] or by camping with

prohibited usage of light-emitting devices,[197] results in an increased daytime exposure to light while preventing nocturnal light exposure. Humans display an advance in their sleep timing under these conditions.[196,197] Melatonin rhythms of campers display even larger phase advances, resulting in an increased time between melatonin onset and sleep initiation and between melatonin offset and awakening.[197] This altered phase angle of entrainment may facilitate falling asleep and awakening and may be particularly advantageous for Evening types, as those scoring towards eveningness display larger camping-induced phase advances.[197] Increased daytime behavioral activity under these conditions may have contributed to the increased amplitude in circadian rhythm (**Chapter 5**).[196,197] Further evidence for benefit of natural light exposure comes from the Amish, a group of traditional church fellowships who do not use artificial sources of light.[198] Interestingly, the Amish have a reduced prevalence of obesity, which may have been caused by changes in diet and activity levels,[199,200] as well as certain types of cancer, even when controlling for tobacco and screening participation.[201]

A robust and high amplitude circadian rhythm is beneficial for sleep. In **Chapter 10**, we describe that short sleeping obese individuals were coached to extend their sleep by implementing sleep hygiene methods. Many of these methods are aimed at improving circadian amplitude, such as avoiding of light at night, increasing daytime light exposure, abstinence from daytime naps and increasing daytime exercise. When these individuals were evaluated averagely 1.3 yrs later, individuals had successfully extended their sleep by average 17 minutes by diary and by 36 minutes by a sleep questionnaire and their sleep quality improved by 23% (**Chapter 10, Table 3**). Individuals also improved in neurocognitive function: mean GDS scores improved by 7%, attention by 10%, and memory and executive functions by 7% and 5% with a borderline significance, respectively (**Chapter 10, Table 2**). It is unlikely that the improvement in neurocognitive performance is caused by practice effects because the time interval between testing was sufficiently large.[202] Additionally, cortisol levels increased by 17% (**Chapter 10, Table 2**). The amelioration in GDS scores associated positively with sleep duration and sleep efficiency (**Chapter 10, Table 5**). It should be noted that since individuals in this study were selected for short sleep, it is also possible that the lengthening in sleep is caused by regression to the mean instead of by true sleep extension. Additionally, only 74 of the 121 participants were evaluated at the final follow up, a rate that is common in prospective studies in obese individuals. Although these participants did not differ in baseline parameters from the participants that were lost due to follow up, follow-up is imperative in any trail and the high dropout rate could have influenced prospective results. Nonetheless, sleep extension and improvement of sleep quality may be beneficial for restoration of neurocognitive function in short sleeping obese individuals. Bright light exposure during the day has also been successful in attenuating cognitive deterioration in nursing home residents.[193]

Taken together, these results imply that detrimental health effects induced by circadian disruption or sleep curtailment are at least partly reversible and that improving circadian rhythmicity and sleep may well be a new target to counteract various negative health effects.

General conclusion and clinical implications

In this thesis, we have demonstrated that high amplitude, well synchronized rhythms are beneficial for health. In the first part of this thesis, we provide more insight in the role of the neurotransmitter VIP and well-timed behavior in achieving such robust rhythms (**Chapter 4, Chapter 6**). Furthermore, we provide evidence that sleep-deprivation-induced suppression of the light response is alleviated by the usage of caffeine (**Chapter 5**). Since light is the most important stimulus that synchronizes us to the environment, a robust light response could assist in synchronizing external and internal rhythms.

In the last parts, we demonstrated the detrimental effects of altered rhythmicity induced by SCN lesions (**Chapter 7**), constant light (**Chapter 8**) and chronotype (**Chapter 9**) on body weight and insulin resistance (**Chapter 7, Chapter 8, Chapter 9**), bones and muscles (**Chapter 8**), immune system (**Chapter 8**), and stress hormones (**Chapter 9**). Additionally, we show an association between sleep loss and bones and muscles (**Chapter 11**) and a decline in neurocognitive function (**Chapter 10**). Diseased states due to a deterioration in these parameters are commonly observed. For example, more than one-third of the US population is obese and the same amount has pre-diabetic levels of glycosylated haemoglobin (HbA1c) (**Chapter 2**), 29% of middle-aged individuals has sarcopenia[203] and 31% has osteopenia.[204] Therefore, establishing additional risk factors is of great importance.

A decline in these health parameters is associated with adverse health outcomes. For instance, a high BMI, a low HDL and insulin resistance predict cardiovascular mortality.[205,206] Insulin resistance also poses a risk factor for non-cardiovascular diseases, like cancer or impaired kidney function.[206] Declined bone mass increases the risk for fractures,[207] which increases risk for mortality[208] and a reduced quality of life.[209] Muscle function is a predictor of functional decline that may occur during aging.[210] Adequate immune responses are essential to battle infection and to protect against cancer, neurodegenerative disease, and auto-immune disease. A pro-inflammatory state has been associated with morbidity and mortality.[211] Likewise, high urinary catecholamine excretion is a risk factor for functional decline and mortality.[212] Declined cognitive function may predispose individuals to errors, accidents, faulty decision making, and ultimately neurodegenerative disease. Declines in health parameters put individuals at increased risk when they occur simultaneously, since buffering capacity is lost and homeostasis in multiple systems is disrupted. Many of these health parameters are strongly correlated and share common determinants.

Results are at particular importance for individuals experiencing internal desynchrony and those at risk for sleep deprivation, such as shift workers, elderly individuals, those experiencing frequent (social) jet lag, and patients suffering from sepsis,[213] neurodegenerative diseases such as Alzheimer,[214,215] and individuals who are exposed to diminished environmental rhythms such as intensive care or nursing homes settings.[193]

Studies described in **Chapter 8** and **Chapter 10** demonstrate that the effects of circadian disruption and sleep loss are reversible. This thesis thus provides new prevention and treatment opportunities for patients suffering from the above mentioned disorders directed towards methods that promote synchrony among SCN cells and entrainment to the environment, like exercise (**Chapter 6**), strong environmental rhythms (**Chapter 8**), and sleep hygiene strategies to ensure a good night's sleep (**Chapter 10**).

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