Circadian timekeeping: from basic clock function to implications for health

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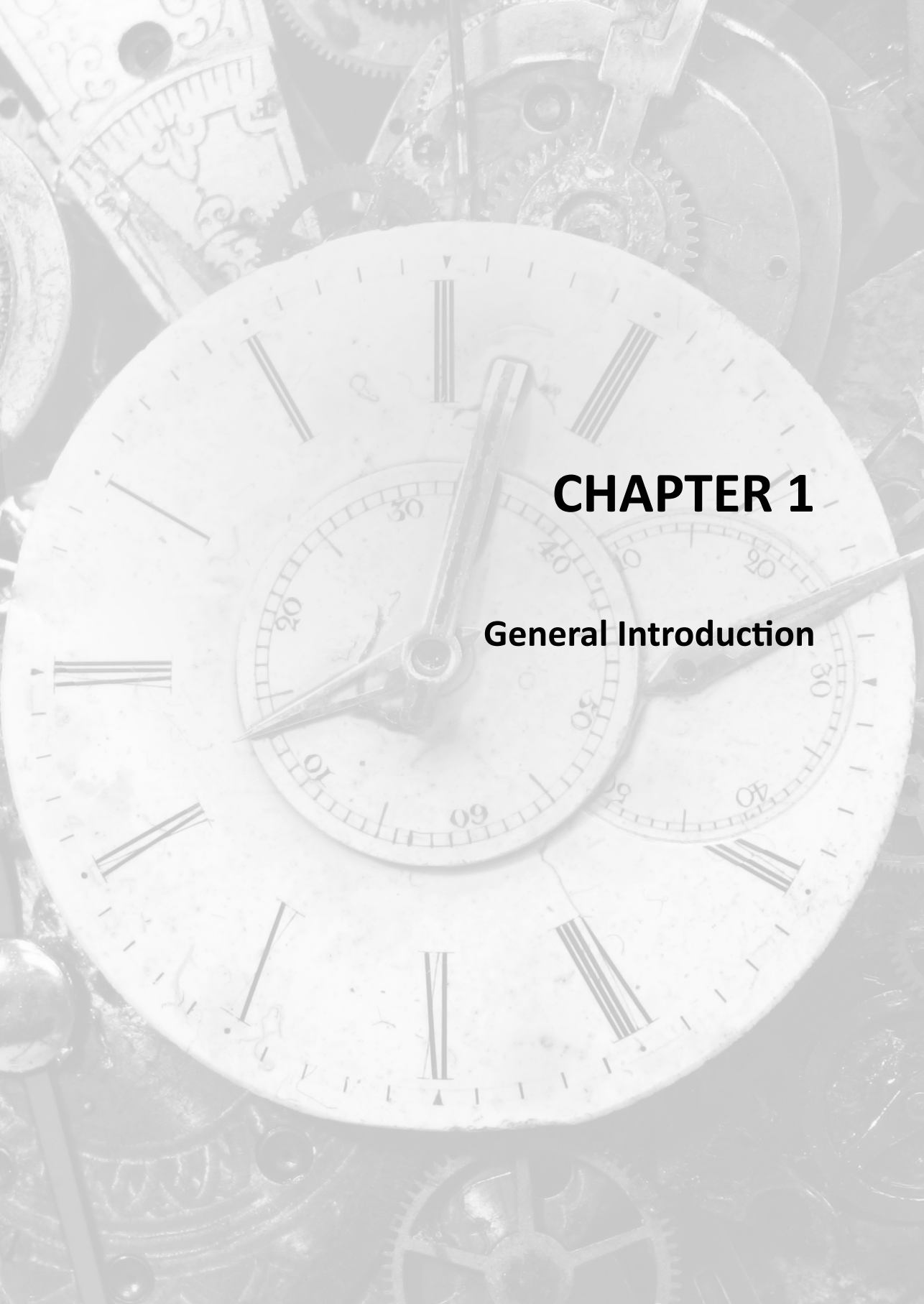

CHAPTER 1

General Introduction

Circadian and seasonal rhythmicity in life on earth

Our natural environment is filled with cyclical patterns whose ultimate origins lie in the configuration of our solar system. Due to the daily rotation of the earth around its axis, the amount of light, temperature, humidity and many other environmental factors differ between day and night. Seasons result from the earth's yearly orbit around the sun with a stable tilt of its rotational axis perpendicular to its orbital plane, which means that one pole will be directed towards the sun at one side of the orbit and half an orbit later this pole will point away from the sun. This leads to strong seasonal differences in temperature, daylength, weather patterns, and food availability, especially at higher latitudes. Organisms on earth need to be constantly adapting to these cyclic variations in environment in order to survive. Diurnal animals reap the benefits of a higher daytime temperature and light exposure, allowing a lower metabolic cost and higher visibility for finding food. Being nocturnal provides the advantage of being harder to detect and avoids competition with diurnal animals. In addition, animals should reproduce in a time of year when there is enough food to feed the offspring, while they should conserve energy in times of food scarcity. It is therefore evolutionary beneficial to have ones metabolism optimalized for active behavior at certain times and rest at other times. Rhythms of about 24 hours, otherwise termed "*circadian rhythms*" (*circa* = around, *dies* = a day),[1] and seasonal rhythms are deeply embedded in evolution. Early life forms that evolved 2.5-3.5 billion years ago, like the prokaryotes *Cyanobacteria*, *Archaea*, and *Proteobacteria*,[2] possess a circadian machinery of separate evolutionary origins in each bacterium.[3] This demonstrates that circadian rhythms were already a key instrument for increasing fitness and survival in early evolution.

In humans, 10% of all gene expression displays circadian patterns of expression and a much larger percentage of the proteome oscillates.[4,5] Consequently, many physiological processes fluctuate throughout the day, including core body temperature,[6] heart rate, insulin sensitivity,[7] energy expenditure, sympathovagal balance,[8] hormone and neurotransmitter secretion, immune function,[9,10] bone remodelling,[11] muscle metabolism,[12] sleepiness,[13] and mental processing speed.[14] These fluctuations allow us to be active and alert during the day and help us sleep at night. Similarly, a wide range of species display yearly cycles of migration and of hibernation: changing their food intake, metabolism and thermoregulation in order to adapt to harsh environmental conditions such as cold or low food abundance.[15]

Circadian rhythms are endogeneously generated and can be entrained

Circadian and seasonal rhythms in these bodily processes are not just a direct response to environmental fluctuations, but they are endogenously generated. The first evidence for this was based on observations of the French geophysicist Jean-Jacques d'Ortous de Mairan.[16] In a letter written in 1729, he describes the opening of the leaves of the *Mimosa pudica* plant during the day and closing during the night. He was intrigued that the daily

opening and closing of the leaves continued in constant darkness, thus in the absence of environmental light input. Since then, many other experiments have been performed confirming the existence of endogenous circadian rhythmicity under constant conditions. The Frenchman Michel Siffre confined himself underground in a cave for 63 days and was the first to prove that circadian rhythms sustained in humans over a prolonged period without environmental cues.[17] Interestingly, Siffre's time of waking became later each day, indicating that his internal rhythm was longer than 24 hrs. In all organisms, rhythms start "*free-running*" in the absence of input from the environment with a period that slightly deviates from 24 hours. Diurnal species will normally free-run with a period longer than 24 hours, whereas nocturnal animals will tend to have a free-running period of less than 24 hours.

Since the earth's day is almost exactly 24 hours, organisms have to adjust to the external cycle on a daily basis. The process of synchronizing the internal rhythm to the environment is called "*entrainment*" and external cues used for entrainment are termed "*Zeitgebers*". This term was first used in 1955 by Jürgen Aschoff,[18] one of the founders of the field of chronobiology. The most important Zeitgeber is light, and other examples are temperature, exercise, social interactions, and temporal food patterns. In 1960, DeCoursey was the first to demonstrate that rodents displayed clear circadian rhythms in behavior when kept in constant darkness, and that the phase of these rhythms could be shifted in response to light.[19] She constructed the first "*phase response curve*", demonstrating that the effect of light depends on time of exposure. Light exposure, or a *photic stimulus*, at the beginning of the night slows down circadian rhythms, resulting in a "*phase delay*" meaning that the animal will wake up later on the subsequent day. Light exposure towards the end of the night will have an opposite effect, speeding up the clock to a "*phase advance*" and thus the animal will start active behavior earlier on the following day. Light pulses during the subjective day, the phase where animals would normally receive light exposure, have no phase-shifting effect on behavior, even when they are kept in constant darkness. The phase response curve to light is very similar in humans, and in the first part of the night, light slows down the pace of our clock, while in the second half of the night light speeds up our clock.[20]

An important *non-photic stimulus* that has the potential to phase shift circadian rhythms is exercise. When animals are given a novel running wheel during the day, this is a phase advancing stimulus.[21] Also other stimuli that evoke behavioral activity, such as cage changing, restricted feeding, dark pulses and sleep deprivation elicit phase shifting effects.[22-26] Certain pharmacological substances that induce behavioral activity can induce phase resetting, such as neuropeptide Y (NPY), serotonin (5-HT) agonists, opioids, and benzodiazepines.[22,23,27-29] In humans, nocturnal exercise can induce phase delays.[30-32] Thus, the phase response curve to non-photic stimuli is out of phase with the phase response curve to light.

Photic and non-photic stimuli can modulate each others phase shifting effects. Non-photic stimuli can block phase shifts that are induced by light. Exposure to 5-HT or GABA receptor agonists, NPY, or wheel running activity blocks light-induced phase shifts at night.[24,33-40] Other stimuli can enhance phase shifting by light. For instance, infusion of NPY- and 5-HT receptor antagonists in combination with a light pulse resulted in very large phase shifts.[41] Conversely, light blocks the phase advances that are induced by NPY, 5-HT agonists, wheel running or sleep deprivation during the day,[42-45] and at the level of the central pacemaker in the brain, light and behavioral activity antagonize each other's effect.[46]

Molecular basis for circadian rhythms

Virtually all cells display circadian rhythmicity that is powered by a transcriptional / translational feedback loop that is hardwired by circadian "*clock genes*" and their protein products. The first clock gene was identified in *Drosophila* and called "*Period*".[47] In 1994, the first mammalian clock gene "*Clock*" (Circadian Locomotor Output Cycles Kaput) was identified by the research group of Joe Takahashi.[48] CLOCK forms dimers with BMAL1[49] that bind to E-box regions of the promotor of clock genes period (*Per1, Per2*)[50] and cryptochrome (*Cry1, Cry2*),[51] thereby inducing their transcription. Subsequently, PERIOD and CRY dimerize and accumulate until their levels are sufficient to inhibit the activity of BMAL1/CLOCK complexes and thus shutting off their own transcription. Over the night, PERIOD/CRY dimers are degraded by casein kinase 1 protein kinases (CSNK1ε, CSNK1δ),[52] permitting the initiation of a new cycle.

A second loop regulates the expression of the *Bmal1* gene. BMAL1/CLOCK dimers also bind to E-boxes initiating transcription of REV-ERBα and RORα. The *Bmal1* promotor region contains a ROR element, that activates *Bmal1* expression when bound by RORα, while REV-ERBα surpresses *Bmal1*. Several clock gene mutant animals have been generated, all displaying abnormalities in their circadian rhythmicity.

The master oscillator in the suprachiasmatic nuclei (SCN)

The anatomical location of the central clock was discovered relatively late in comparison to accumulating evidence of general properties of circadian rhythms. As light is the most important Zeitgeber for circadian rhythms, the central clock was hypothesized to lay in the vincity of the optic tract. In 1972, a small pair of structures located in the anterior hypothalamus was identified that when lesioned resulted in a complete loss of circadian rhythms.[53,54] The hypothalamus is an evolutionarily ancient part of the brain that is responsible for regulating key physiological processes such as metabolism, body temperature, hunger, thirst, reproduction, thereby maximizing survival and fitness. The brain structures responsible for circadian rhythms were located just above the optic chiasm, so they were termed the "*suprachiamatic nuclei*" (SCN). The final establishment of the

SCN as the circadian pacemaker was made in 1990 when SCN lesions were made in normal hamsters and the SCN of a clock gene mutant hamster with a shorter free running period was transplanted into the lesioned site of the normal hamsters.[55] Interestingly, the SCN-lesioned hamsters now displayed a circadian rhythm with the shorter free running period of the transplanted SCN, demonstrating that the period of the behavioral circadian rhythm is determined by the SCN.

Inputs of the SCN

Light reaches photoreceptive rods and cones in the retina, which contain rhodopsins and photopsins and change their membrane potential in response to light input. They are connected to the visual cortex via the optic nerves and are essential for vision. Recently, melanopsin-containing ganglion cells were discovered in the retina, which convey information for accessory visual functions.[56] These cells play a key role in circadian photoentrainment, as mice without rods and cones retained the ability to synchronize to light-dark cycles.[57,58] While rods and cones become saturated by prolonged light input bleaching their response, melanopsin-containing ganglions gradually increase their activity reaching a sustained level, which makes them more appropriate sensors measuring light-input. In this system, light is transmitted through the retino-hypothalamic tract (RHT) to the SCN, the intergeniculate leaflet (IGL) and the olivary pretectal nucleus (OPN). Neurons composing this tract release pituitary adenylate cyclase-activating peptide (PACAP) and glutamate upon light exposure, increasing the firing rate of SCN neurons and increasing Ca^{2+} concentrations through activation of glutamate receptors and voltage-sensitive calcium currents.[59-61] The net result is transcription of *c-Fos*, *Per1* and *Per2*. The IGL projects to the SCN with fibers that produce neuropeptide Y (NPY) in response to photic stimuli and thus enhances the light-responsiveness of the SCN.[62]

The terminals of the brainstem raphe nucleus release 5-HT in the SCN whenever animals are active.[63,64] 5-HT modulates light-induced glutamate release from the RHT and alters the postsynaptic sensitivity to glutamate, hereby changing the sensitivity of SCN neurons to light.[33,35] The IGL terminals also release NPY in response to behavior and NPY release is enhanced by 5-HT.[65] Through this pathway, behavior affects the SCN. Behavioral activity during the day leads to a suppressed firing rate of SCN neurons *in vivo*.[46,66] *In vitro* application of NPY and the 5-HT A/7 receptor agonist 8-OH-DPAT also suppresses SCN neuronal activity.[67-69] NPY application onto the SCN *in vivo* blocked novelty-induced phase shifts in wheel running.[70]

The SCN also posseses receptors for several other substances, including melatonin,[71,72] androgens,[73] cytokines like interferon-α and tumor necrosis factor-α (TNF-α)[74,75] that are capable of phase-shifting the SCN.

Neuronal activity of the SCN – *united we stand, divided we fall*

In 1979, the multiunit neuronal activity of the SCN was first recorded *in vivo* in rats.[76] Electrodes were placed in the brain and targeted at the SCN, showing that SCN neurons display most action potentials during the day and are less active during the night. This rhythm persisted in the absence of light and also when connections with the rest of the brain were surgically cut, delivering additional proof that the SCN is an endogenous and autonomous pacemaker. SCN neuronal actitivy was also measured *in vitro* in rat brain slices that were articificially kept alive, also showing high multiunit activity (MUA) during the day and low MUA at night.[77-79] The SCN are compromised of about 20,000 neurons that individually generate spontaneous action potentials of 10-15 spikes per second for about three to six hrs a day, while they are silent for the remaining hours.[80-84] This functionality persists when SCN neurons are extracted from the brain and cultured *in vitro*, demonstrating that this is a cell-autonomous property.[85] This circadian function is explained by the fact that the SCN neuron cell membrane is spontaneously depolarized for several hrs each day, because several ion channels modulating membrane potential are under circadian control.[61]

SCN cells have the unique property to communicate temporal signals to each other by coupling the molecular clock to the membrane potential. Neurons are directly connected through synapses, allowing coupling through neurotransmitter release. Some SCN neurons are also coupled by gap junctions, connections that allow various molecules, ions and electrical impulses to directly pass through regulated intercellular gates. Each individual SCN neuron is imprecise and variable in period, with most but not all SCN neurons spiking during the day for somewhat various durations. However, the SCN network as a whole produces a robust rhythm. Even in clock gene mutant mice that display rhythm abnormalities the SCN displays a robust multi unit rhythm, showing that the network can rescue defects in individual cells. Communication of the SCN cells through synapses and gap junctions allows synchronization by constant adjustment of the phase of their rhythms. This results in a robust and high amplitude output rhythm of the SCN, informing the body about the time of day.

The time of year is also reflected in the MUA pattern of SCN. While individual neurons only spike for some hrs per day, the MUA rhythm of the SCN is high troughout the day. Interestingly, when days are longer, the MUA activity remains high throughout the phase of light exposure.[83] In other words, in long photoperiods, the electrical activity pattern is broad. Equally, in short photoperiods the MUA peak narrows. The duration of a single neuron spiking does not depend on day length. Instead, the phase distribution of the time of spiking differs between long and short days. When mice are placed in constant darkness after long and short days, the waveform of the MUA still reflects the duration of the preceding day. This proves that the broadening and narrowing of the peak in response to longer and shorter days is not just a direct response to light, but that photoperiodic information is actually

stored and used by the SCN. [83] In this manner, the SCN provides the body with seasonal cues.[86,87] The neurotransmitter vasoactive intestinal peptide (VIP) has been proposed as a major substance involved in this process (**Chapter 4**).

Regional differences within the SCN

The SCN can functionally be divided into two main parts: the core and the shell region.[88] The exact anatomical delineation between them differs between species and is not always sharp,[89] but the two regions differ substantially in afferent and efferent connections and in peptidergic phenotype. The core region is densily innervated by retinal ganglion cells from the RHT which release glutamate and PACAP upon light exposure. Many serotonergic fibers from the raphe and NPY fibers from the IGL also terminate in this part. It is rich in VIP- and gastrin-releasing peptide (GRP)-containing neurons.[80] Hence, this region appears to gather environmental information in order to synchronize internal to external rhythms.

The shell region mainly contains arginine vasopressin (AVP)-producing neurons, which is expressed in a rhythmic manner, even in the abcence of external stimuli.[90,91] It is not retinorecipient and appears to be mainly important to communicate temporal information to downstream targets. When animals are exposed to a phase shift of the light schedule, the neuronal activity rhythm in the core region adapts immediately, while the shell region takes several days to adjust.[92,93]

When the core and shell regions are surgically separated *in vitro* after exposure to a phase shift, the shell region will not entrain to the new rhythm, implying the need for communication between the two regions for mutual synchronization. GABA and VIP are major players in this process and blocking GABA-A receptors after a phase shift will have the same resuls as surgical separation of the shell and core regions: the shell region will not adapt to the new phase.[92,94] Likewise, VIP-deficient or VIP2R-receptor deficient mice fail to display robust circadian rhythms under constant conditions, suggesting a role for VIP in entrainment.[95-98]

Output pathways of the SCN

The SCN produces both neuronal and humoral output and there is evidence that both mechanisms are functional.[99] The bulk of SCN neurons project to the subparaventricular zone (sPVZ) and the dorsomedial hypothalamic nucleus (DMH). The sPVZ is a major relay station in the output of temporal information and contains a ventral and dorsal part. Lesions of the ventral sPVZ disrupt sleep-wake and behavioral rhythms, whereas a lesioned dorsal part results in severely disrupted rhythms of body temperature.[100] Two major output areas of the sPVZ are the medial preoptic region (MPO), which controls circadian rhythms of body temperature, and the DMH.[101-103] The DMH relays SCN output to the paraventricular nucleus (PVN), which controls daily hormonal secretion; the lateral hypothalamus (LH), regulator

of sleep-wake and feeding cycles; and the sleep-regulating ventrolateral preoptic nucleus (VLPO).[102] The DMH-VLPO connection largely consists of GABA-containing neurons that inhibit sleep upon activation, whereas the neurons in the projection to the LH contain mostly glutamate and thyrotropin-releasing hormone which promote wakefulness. Lesions of the DMH result in increased sleep and decreased behavioral activity, implying that the DMH is mainly activating.[104] The DMH seems to integrate temporal information from the SCN and the sPVZ in order to optimize adaptation to the environment and thereby benefit survival.

Interestingly, in both diurnal and nocturnal animals, SCN neurons are most active during the light period. Since the VLPO is active during sleep in all species,[105] the relay from SCN to VLPO must differ in diurnal and nocturnal animals. On the other hand, diurnality or nocturnality is not always an entirely fixed characteristic. For example, when access to food is restricted to the daylight hrs in mice, these normally nocturnal animals will display most behavioral activity during daylight hours, thereby acting as if they are diurnal animals.[106] This rhythm persists for several days in the absence of food input. This suggests that the neuro-anatomical differences between diurnal and nocturnal species are plastic.

The SCN innervates the pineal gland through a multi-synaptic pathway involving the PVN, the intermediolateral cell column of the spinal cord and the superior cervical ganglion.[107-109] The pineal gland produces melatonin in the absence of light exposure and transfers this temporal information to a multitude of tissues expressing melatonin receptors.[110] In a comparable way the SCN also controls the daily rhythm of corticosterone release from the adrenal gland[54] and many other hormonal rhythms (**Chapter 2**).[111,112] Thus, these hormones may also serve to transfer temporal information to peripheral tissues.

The SCN can transfer temporal information even in the absence of synaptical connections. When SCN tissue from normal hamsters with a 24 hr period is placed in a semipermeable polymer capsule in SCN-lesioned mutant hamsters that originally had a period of 22 hours, these mutant hamsters display a circadian rhythm of 24 hrs.[113] This study proved that direct axonal connections were not necessary for the SCN to transfer a sustainable rhythm in behavior. Several humoral substances have been implicated in the transferral of rhythmic information: transformin growth factor alpha (TGF-α), prokinectin 2 (PK2), cardiotrophin-like cytokine (CLC), and AVP.[114-117] These diffusables are rhythmically produced by the SCN and injection into the SCN or third ventricle influences behavioral activity. However, also clear evidence was provided that if neuro-endocrine rhythms were to be sustained point-to-point neuronal connections are necessary.[118,119]

Partially independent circadian oscillations are also present outside the SCN in many organs and cells throughout the body, and are referred to as "*peripheral clocks*".[120] A transgenic mouse line was developed with the enzyme luciferase bound to the promotor region of clock gene *Per1*. This coupling leads to light emission upon transcription of *Per1*. Yamazaki and colleagues demonstrated a rhythmic light emission from the SCN *in vitro,*

hereby showing rhythmicity of *Per1* transcription. In addition, rhythmic *Per1* expression was present in liver, lung, and skeletal muscle tissue *in vitro*, but rhythms dampened after 2 to 7 cycles. SCN lesioning prevented rhythmic cycling of peripheral tissue oscillators.[121] Thus, isolated tissue cannot sustain robust rhythmic oscillations without the input of the master pacemaker in the SCN. The SCN entrains and synchronizes the periphery through direct and indirect dues. The SCN appears to control peripheral clocks directly through humoral signals, like cyclically secreted hormones such as glucocorticoids, and more indirectly via feeding and body temperature rhythms which in turn are partly driven by the SCN directed rest-activity patterns.[122] In response to a phase shift, the SCN adapts to the new phase relatively fast, whereas the peripheral oscillators shift to the new phase in the course of a few days.[120,123]

Physiology of sleep

A normal night of sleep consists of series of sleep stages in which different brain wave patterns are displayed. Roughly, it is divided into rapid eye moment (REM) and nonrapid eye movement (NREM) sleep. Most dreaming occurs during REM sleep, when most skeletal muscles are hypo- or atonic. NREM can be further subdivided into 4 distinct sleep stages. Stage 1 is relatively light sleep in which high amplitude theta waves, very slow brain waves, can be observed. Sleep spindles begin to appear in stage 2, bursts of rhythmic brain wave activity. The deeper sleep stages 3 and 4 are characterized by slow brain waves known as delta waves and therefore also termed slow-wave sleep (SWS). As the night progresses and sleep pressure is declining, less NREM and more REM sleep is displayed.

Although sleep and circadian rhythms are tightly linked in everyday life, sleep is not merely regulated in a circadian manner. Apart from the circadian influence on sleep and wakefulness, there is an additional mechanism regulating sleep pressure: the homeostatic drive for sleep. The terms "*process C*" and "*process S*", respectively, have been proposed for these processes by Alex Borbély.[124,125] The homeostatic drive progressively accumulates sleep pressure during prolonged wakefulness and sleep propensity is reduced with sleep. Process C is a circadian function that dictates wakefulness during the day and sleep at night in diurnal species. When an individual is sleep deprived, sleepiness will still fluctuate over the course of day and night. Sleep pressure at any time results from the interaction of these two processes.

The first postulations of the localisation of the sleep-wake regulatory system were made by von Economo in 1930.[126] He observed that patients with encephalitis lethargica, a viral disease causing lesions in the posterior hypothalamus and rostral midbrain, slept for 20 or more hrs a day for many weeks. A subset of patients, namely those with lesions involving the basal ganglia and anterior hypothalamus, could not sleep longer than a few hours, despite being extremely tired. It has since then been confirmed that alertness consists of two major ascending pathways stimulating forebrain and cortical arousal. The

first pathway innervates the thalamus and the second one passes through the posterior hypothalamus and the forebrain. The pathways involve multiple other brain areas, including the laterodorsal tegmental nucleus (PPT/LDT), locus coeruleus, median raphe nucleus, and tuberomammillary nucleus (TMN). A group of neurons in the VLPO is important for quieting the ascending arousal system during sleep.[127,128] These neurons project to monoaminergic systems in the hypothalamus and brainstem and contain the inhibitory neurotransmitters GABA and galanin. When this area is damaged, sleep is decreased.[129] The inhibitory action is bidirectional, as activity of monoamine nuclei during wakefulness also decreases VLPO activity.[130] The switch between wakefulness (active arousal system and inactive VLPO) and sleep (inactive arousal system and active VLPO) is binary, and partial on or off states are avoided. Therefore, alternations between sleep and wake states are relatively quick. The neuropeptide orexin, produced in the lateral hypothalamus and mainly present in the ascending arousal system, may provide additional stability for the on or off switch.

Adenosine acting in the basal forebrain is a key regulator of sleep homeostasis.[131,132] Glucose is stored as glycogen. During the breakdown of glycogen and subsequent oxidation of glucose (glycolysis), adenosine triphosphate (ATP) is formed. Adenosine is a breakdown product of ATP. During wakefulness, ATP is used as energy supplier, causing the levels of adenosine to rise in parallel with accumulation of sleep pressure.[133] With rest occurring during sleep, ATP stores are replenished.[134,135] Injection of adenosine in the basal forebrain of animals promoted sleep via de adenosine receptor A1 by both inhibiting the basal forebrain arousal system and exciting VLPO neurons.[136,137]

Feedback of sleep on the SCN

SCN activity is influenced by sleep stages and by components of the sleep system. REM sleep associates with high SCN activity and NREM with low SCN activity.[138] Stimulation of the PPT/LDT of the ascending arousal pathway influences neurotransmitter levels in the SCN.[139] Furthermore, electrical activation of this pathway can induce phase shifts in behavioral activity.[139]

Circadian regulation is affected by sleep deprivation. Hypothalamic 5-HT turnover and 5-HT release in the SCN is increased in response to sleep deprivation.[43,140] Sleep deprivation also reduces glucose usage in several brain areas, including the VMH, which could affect SCN function.[141] In addition, neuronal firing in the SCN decreases in response to increased homeostatic sleep pressure induced by sleep deprivation.[125,138,142]

Sleep deprivation decreases phase shifts elicited by light, as well as light-induced *c-Fos* expression in the SCN.[143,144] Infusion of adenosine receptor agonists produces a similar effect.[145-147] The substances 5-HT, adenosine and glucose are candidates that may be responsible for the altered light response in sleep deprived animals.[144]

Disrupted rhythms and sleep in society

Many individuals in the modern world are subject to disruption of their sleep-wake cycle. Artificial lighting as well as economic demands have contributed to a 24-hour society in current times, further supported by technological profileration and globalisation of economy. Recent satellite data revealed that 75% of the US and European population is exposed to light at night.[148] Additionally, medical environments such as intensive care units and nursing homes exhibit little diurnal variability in light levels.[149,150] Animals exposed to constant light depict decreased or absent rhythms in behavior, glucocorticoids and body temperature.[151-156] This is caused by desynchronization of clock neurons in the SCN.[157] A dim level of light at night also attenuates rhythmic expression of clock genes,[158,159] but does not seem to disturb glucocoirticoid or behavioral rhythmicity.[151,159]

About one third of individuals in the US reported sleeping less than 6 hrs per night, considerably shorter than the 7-9 hrs that is recommended.[160] Additionally, sleep *timing* is frequently unnatural or unstable. Twenty percent of the working force is engaged in shift work, a state where behavior is often discrepant with internal circadian rhythms.[161] With the introduction of air travel and growing of transmeridian air traffic, an increasing number of individuals is exposed to rapid changes in time zones and experience jet lag. Jet lag results from a desynchrony between the SCN and peripheral oscillators in the body, since these take different times to phase shift and adjust to the new day-night rhythm.[120,123] Another common phenomenon is "*social jet lag*", which does not result from shifting time zones but refers to a discrepancy of sleep timing on working days compared to the weekends due to differential social demands.[162] Chronic environmental desynchrony can disrupt molecular clock mechanisms in peripheral cells.[163]

Caffeine is often used to counteract internal signals of sleep pressure in order to keep up with external demands. Caffeine is an adenosine receptor antagonist that disrupts sleep and increases altertness.[164] The food and drug administration reports a mean daily intake of 300 mg of caffeine in the US.[165] Coffee drinkers averagely consume 3.3 cups per day. In humans, caffeine attenuates the decrease in body temperature in the evening, especially when combined with light, suggesting an effect on light sensitivity (**Chapter 5**).[166]

Health effects of insufficient sleep and disrupted rhythms

Insufficient sleep has been related to a spectrum of cardiovascular and metabolic diseases by altering multiple pathways (**Chapter 1, Chapter 2**). In addition, insufficient sleep may be a risk factor for osteoporosis and sarcopenia, two closely related conditions that puts individuals at risk for falls, fractures and disability.[167,168] Sleep deprivation also interferes with mental functioning, such as attention and memory,[169-171] resulting in impairments in clinical performance and more accidents.[172] Thereby, inadequate sleep appears to increase frailty: a clinical state in which there is an increase in an individual's vulnerability for increased dependency when exposed to a stressor.[173]

Shift workers are more prone to metabolic disturbances, such as obesity[174] and diabetes,[175] two conditions that increase cardiometabolic risk. They have higher markers of inflammation[176] and may also have an increased risk for cancer: a meta-analysis reported an association between breast cancer risk and nightwork.[177] In addition, shift workers have decreased bone mineral density[178] and a higher risk for bone fractures.[179]

Short term experimentally-induced discrepancy between external and internal circadian rhythms may already negatively affect cardiometabolic health. A 10-day circadian misalignment protocol (28-hr days) which caused participants to eat and sleep out of phase from their habitual times resulted in increased blood pressure and decreased glucose tolerance.[180] Forced circadian disruption also leads to changes in glucose metabolism, a larger body mass[181] and increased risk for cardiometabolic disease.[182]

Chronic jetlag exposure in rodents causes a higher body mass,[183] impairs insulin secretion,[184] results in higher levels of pro-inflammatory cytokines and lower levels of anti-inflammatory cytokines,[185] alters circadian rhythms in natural killer (NK) cells,[186] promotes tumor progression and metastasis[186-188] and increases mortality.[189] Cell cycle related genes are differentially activated by chronic jet lag resulting in disturbed apoptotic function in mice, demonstrating that circadian disruption can directly effect expression of our genetic material.[187] Chronic exposure to phase advances appears to be more detrimental to health than repeatedly phase delaying animals: 68% of elderly mice survived 8 wks of repetitive delays, compared to 47% of advancers.[189] Disruption of circadian rhythms also affects mental health, as chronic jetlag exposure results in spatial cognitive deficits.[190,191]

Light exposure at night also results in a range of undesirable health effects, such as altered cognitive and affective responses,[192-194] increased weight gain,[152,195] higher blood pressure,[196] a pro-inflammatory state,[195] impaired immunity,[192,197,198] promoted tumor growth,[199-201] and even increased mortality.[201]

Negative health effects of disturbed rhythms may be reversible. For example, restoration of dark nights after a 4-week exposure to dim light at night ameliorated metabolic disruption in mice.[202] Additionally, cognitive function in ex-shift workers slowly recovered over the course of five years.[203]

Chronotype

The individual characteristic that defines preference for sleep timing is called "*chronotype*". morning types are most likely to be most active during the early hours of the day and tend to go to bed early, while evening types prefer being active at later times of the day. In 1976, a questionnaire was developed by Horne and Östberg, consisting of 19 questions about preferred sleep and behavior timing.[204] This questionnaire has been widely used to assess chronotype quantitativily. The chronotype distribution is Gaussian, with only few individuals displaying extreme morning or evening chronotype behavior, and a mean mid-sleep time of 04:30 on free days.[205]

Chronotype is a stable trait that is independent of socio-economic status, gender and etnicity.[206] Chronotype does vary with age: in the early adult years a peak in evening preference is reached, then chronotype progressively moves towards morningness.[205] A heritability of 43-57% has been reported by means of twin studies.[207-209] Polymorphisms in circadian clock genes have been associated with diurnal preference.[210-213]

Additionally, it has been hypothesized that chronotypes have differences in their homeostatic sleep drives, with morning types accumulating and dissipating sleep pressure faster and therefore favouring earlier wake- and bedtimes.[214-216] A month long study where individuals were exposed to a forced desynchony protocol, thereby revealing their intrinsic period, found that morningness correlates with a shorter period of core body temperature rhythm.[217] However, periods only mildly differ with a mean period of 24.1 hrs in morning types and 24.3 hrs in evening types, so this difference would not fully explain the differences in sleep timing between chronotypes. Skin fibroblasts from morning types also cycle with a slightly shorter circadian period (morning types: 24.3 hours; evening types: 24.7 hours).[218] The phase shifting capacity of their fibroblasts also differs: the morning type fibroblasts display larger phase advances and delays in response to *in vitro* aplications with forskolin.[218]

The phase angle of entrainment is the relationship between the external environment and the internal rhythm. Differences between chronotypes in the phase angle of entrainment to a light-dark cycle have been reported. In a laboratory environment, the phase of melatonin, core temperature rhythms and usual wake up time was earlier in morning types.[217] The temporal difference between phase of bodily rhythms and wake up time was longer in morning types, meaning that they woke at a later internal circadian phase compared to evening types. Comparably, maximal subjective sleepiness levels occurred at a much earlier phase for morning types, 6-9 hrs before evening types, while core body temperature and melatonin levels only differed about 2-3 hrs between chronotypes.[219] Outside the laboratory, similar differences in phase angle of entrainment have been reported, with morning types sleeping at a later phase of their body temperature and sleep propensity.[220-222] Possibly, this is due to social factors: morning types have to delay their bedtime to meet social demands, while evening types have to wake up earlier than preferred.

Chronotype is also a self-enforcing trait through entrainment of the clock. For example, morning types are exposed to light at an earlier time of day, thereby entraining their SCN to an earlier time than evening types.[223] Also other entraining stimuli, like food intake, are shifted to an earlier time of day in morning types. Other behavioral differences could also contribute to other sleep timing in chronotypes. It has also been reported that morning and evening types express different personality traits, with evening types displaying more novelty-seeking behavior, more anxiety and worse school performance.[224-226] They also have more sleeping problems and engage in substance abuse more often.[226] Evening types also differ from morning types somatically, with a tendency for higher BMI, which may be

caused by lower dietary constraint and a less healthy diet,[227-228] and a higher risk for type 2 diabetes and hypertension.[229] While differences in behavior may be favoured by different sleep timing, sleep timing preference could also be the consequence of behavior**.**

Outline of this thesis

The first Chapters of this thesis contain two reviews describing the interplay between sleep deprivation and metabolic disturbances (**Chapters 2 and 3**). The experimental part of this thesis is divided into three parts, beginning with basic clock mechanisms and then becoming progressively clinical. Part of the experiments described in this thesis have been performed in mice (**Chapter 4 to 8**), whereas another part of the experiments hase been performed in a human study population comprised of either obese short sleeping US citizens (**Chapters 9 and 10**) or Dutch individuals (**Chapter 11**). Furthermore, most experiments have been performed in the Leiden University Medical Center (LUMC) in the Netherlands (**Chapter 4 to 8** and **Chapter 11**) and two projects were executed at the National Institutes of Health (NIH) in the United States (**Chapters 9 and 10**).

PART I - The basic functioning of the circadian pacemaker

In **Chapter 4**, we investigated the role of the neurotransmitter VIP in synchronization of SCN neurons. Therefore, we examined behavioral and neuronal activity patterns in mice lacking VIP. We implanted micro-electrodes in the SCN of mice to record the MUA *in vivo* and we used passive infrared sensors (PIR) and wheel-running sensors to record behavioral activity. First, we performed a detailed analysis of MUA and behavioral patterns in VIP KO and control mice in a light-dark environment, and also in constant darkness conditions so that the endogenous rhythm of the SCN without environmental input becomes visible. Then, we repeated these analysis in animals that were exposed to short and long photoperiods to simulate winter and summer days. SCN neurons have a different phase distribution depending on photoperiods which is normally sustained in constant darkness.[83] Thus, by observing VIP KO mice in short and long photoperiods, we could observe whether VIP is important in plasticity of the phase distribution of SCN neurons and whether these differences would be maintained without photic input.

Chapter 5 describes experiments investigating the role of caffeine in light responsiveness of the SCN of sleep-deprived mice. Sleep deprivation increases adenosine levels and decreases the phase shifting capacity of the SCN in response to light.[143,144] Adenosine agonists and antagonists reduced and enhanced light-induced phase shifting, respectively.[145-147] Caffeine mainly acts as an adenosine receptor antagonist.[164] Since sleep deprivation and caffeine consumption often occur in conjunction in humans, we examined the combined effect of caffeine and sleep deprivation on circadian regulation. We determined the magnitude of the

phase shift in behavior in response to light in mice with- and without sleep deprivation and the phase shift caused by sleep deprivation alone. In the SCN, we observed direct responses in neuronal activity to light pulses in non-sleep-deprived mice, in sleep-deprived mice with control injections and in sleep-deprived mice that received caffeine injections. Actograms were made of mice in regular light-dark cycles, in constant darkness (to reveal endogenous circadian activity), and in constant light (to observe endogenous circadian activity in combination with constant light input). These mice were given caffeine in their drinking water, and we determined the effects of caffeine on amount of total behavioral activity and on the period of their free running activity.

The experiments described in **Chapter 6** investigate the relationship of behavioral activity and SCN functioning. Timing of behavioral activity is in large part dictated by the SCN. *Vice versa*, the behavioral activity influences the circadian system, as it can induce phase shifts or period changes.[23,230,231] Spontaneous behavioral activity is accompanied by an immediate suppression of SCN activity. We examined the direct relationship between mouse behavior and neuronal activity levels in the SCN in detail. Moreover, through simultaneous video analysis and MUA recordings, we compared the relationship of type and intensity of behavior and changes in SCN firing rate. Additionally, by mild behavioral manipulations we examined the SCN response to induced rather than spontaneous behavior.

PART II - Health outcomes of altered circadian rhythms

In **Chapter 7**, we present studies that examined the role of disturbed function of the SCN in obesity and type 2 diabetes. A link between circadian rhythms and metabolism has been suggested from clock gene mutant mice. For example, *Clock* gene mutant mice are hyperphagic, obese, and hyperglycemic,[232] indicating a link between circadian rhythms and metabolism. However, by the use of these models it is not possible to distuinguish the contribution of the SCN to the metabolic effects, because clock gene mutations are not specific to the SCN. SCN rhythmicity is altered in aging[233] and neurogenerative disorders.[61,234] By performing bilateral SCN lesions in mice, we examined the effect of dysfunction of the central clock on metabolic parameters, including body mass and composition, indirect calorimetry measuring oxygen consumption, food intake and activity in metabolic cages, and hyperinsulinemic-euglycemic clamp studies in order to assess insulin sensitivity. Additionally, some lesions induced collateral damage to areas surrounding the SCN that regulate energy homeostasis, such as the PVN and the VMH, so that the combined effects of loss of the central clock and damage to these areas could be assessed separately.

Another, less invasive method that we used was exposure of mice to constant light and is presented in **Chapter 8**. Hereby, we mimick the overuse of artificial light in modern society,[148] the absence of environmental temporal information and the following

attenuation of circadian rhythmicity.[157] Light exposure at unnatural phases decreases photosynthesis, growth and survival in plants,[235] demonstrating the detrimental health effects of a desynchrony between environmental and internal rhythmicity. Human studies show an association between shift work and loss of health,[174-179] but cannot distinguish cause and effect and the selective contribution of light exposure at night. Therefore, we examined the long term health effects of light at night in a large multidisciplinary and strictly controlled study in mice. In addition, we assessed the reversibility of these effects upon restoration of the normal light-dark regime. Well functioning muscles, strong bones, and adequate immune system responses are main contributers to health and fitness. Therefore, we assessed muscle function, bone microstructure, immune response and homeostasis at several timepoints of circadian disruption (2, 8, 16 and 24 wks) and after subsequent restoration of regular 12 hr light; 12 hr dark cycles.

Beside deliberate pertubations of circadian rhythms, differences in health outcomes may also arise from individual variations in circadian systems. In contrast to experimental mouse studies described above, studies in **Chapter 9** describe cross-sectional analyses in a human study that compares metabolism in morning and evening types. The cohort consisted of obese, short sleeping US individuals and was orginally set up for the *Sleep Extension Study*.[236] While it is known that morning and evening types differ in certain health parametes, like BMI and risk for type 2 diabetes and hypertension,[227-229] the effect of chronotype on metabolic parameters in obese and short sleeping individuals has not been described. Demographic, anthropometric, sleep and food intake characteristics were compared between chronotypes. Furthermore, we examined differences in endocrine features (plasma adrenocorticotropic hormone (ACTH) levels, plasma cortisol levels and urinary norepinephrine and epinephrine levels) and metabolic features (plasma insulin, glucose and lipid levels) between morning and evening types. Multivariate statistical models were constructed to assess the independent importance of parameters in chronotype.

PART III – Health outcomes of insufficient sleep

In **Chapter 10**, we describe studies that analyzed the same human cohort of short sleeping obese American individuals as used in **Chapter 9**, but also included longitudinal analyses. Short sleep and obesity are both correlated with neurocognitive defects. Sleep deprivation mainly affects attention and memory functions, while obesity predominantly interferes with more complex tasks in the executive domain.[169,237-242] Their combined effects on neurocognitive function has never been evaluated. Therefore, we assessed performance of our participants on a neurocognitive battery that included tests in the memory, attention, motor skills and executive function domain. We compared demographic, anthropometric, sleep and hormonal characteristics of participants with a neurocognitive score indicating

global deficits or deficits in specific neurocognitive domains. Subsequently, we examined whether neurocognitive deficits were reversible by sleep extension, achieved by personalized coaching. To accomplish this, neurocognitive functions, sleep and hormonal parameters, were reassessed averagely 1.5 yrs later and compared to values at baseline. Furthermore, mixed models were constructed to assess which parameter influenced neurocognitive performance most strongly.

Sleep characteristics have also been associated with musculoskeletal health, but studies are scarce and inconsistent.[243-246] Osteopenia (low bone mineral density, BMD) and sarcopenia (low muscle mass) are common problems in the elderly that induce a state of vulnerability,[245-246] so risk factors need to be identified. In **Chapter 11**, we evaluated the associations between sleep parameters and osteopenia/sarcopenia in the study population of the *Netherlands Epidemiology for Obesity (NEO) study*[247] (45-65 yrs old, 56% women, BMI 26 ± 5kg/m^2) in further detail. As a lack of sleep is known to affect certain mediators that are potentially harmful to bones and muscles such as cortisol and catecholamines, we hypothesized that insufficient sleep would associate with osteopenia/sarcopenia. As the "restorative" effect of sleep is dependent on sleep duration, but also on sleep quality and sleep timing, we included these sleep parameters in our analyses. Furthermore, we included stratified analyses for sex and menopausal status, and determined whether the associations of sleep with osteopenia/sarcopenia were independent of muscle mass and BMI, respectively.

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