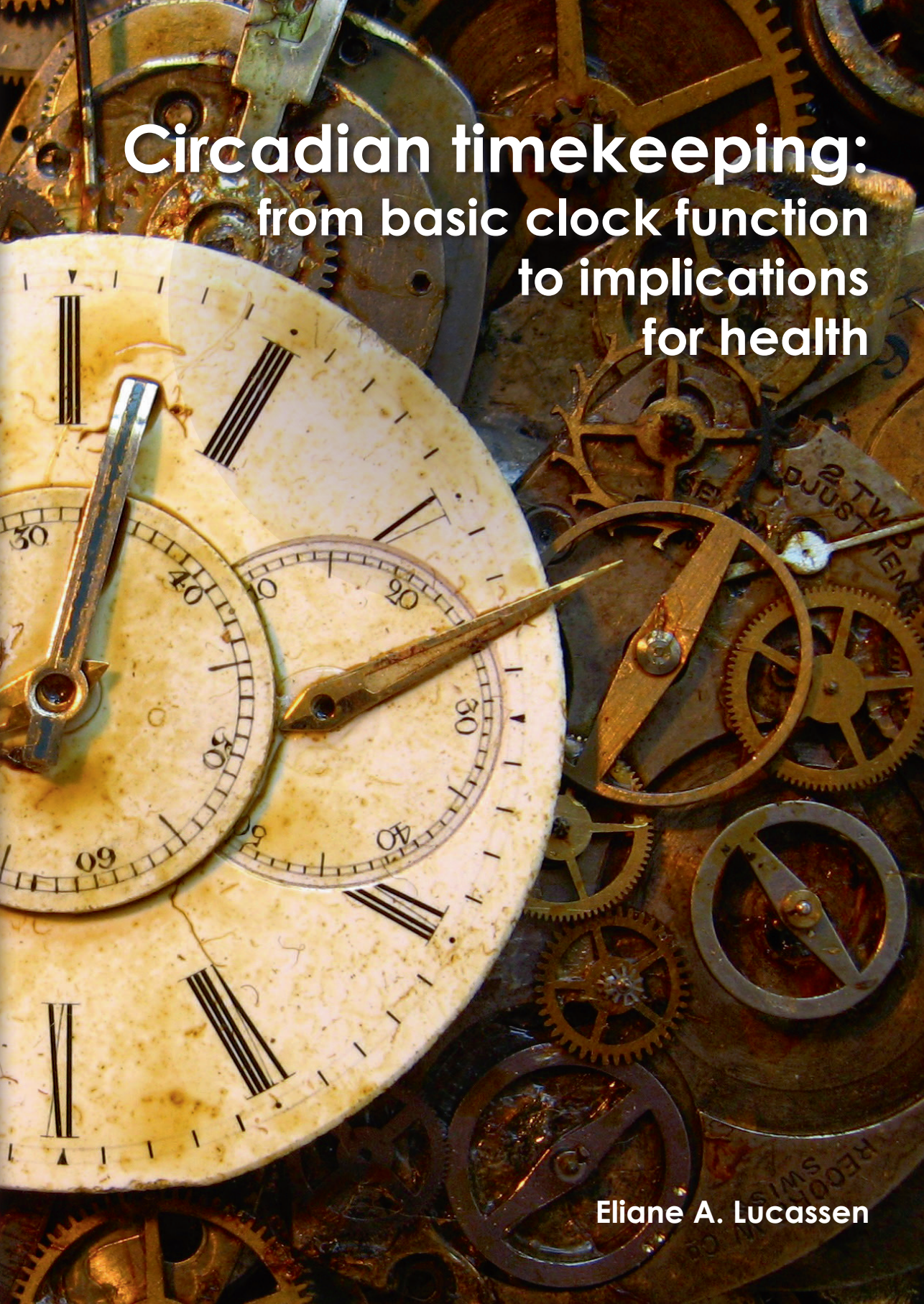
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Eliane A. Lucassen

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Circadian timekeeping: from basic clock function to implications for health

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Promotor
Prof. dr. J.H. Meijer

Copromotor
Prof. dr. A. Kalsbeek (Amsterdam Medical Center)

Promotiecommissie
dr. A.M. Aartsma-Rus
Prof. dr. E. van Cauter (University of Chicago)
Prof. dr. A.J.W. van der Does
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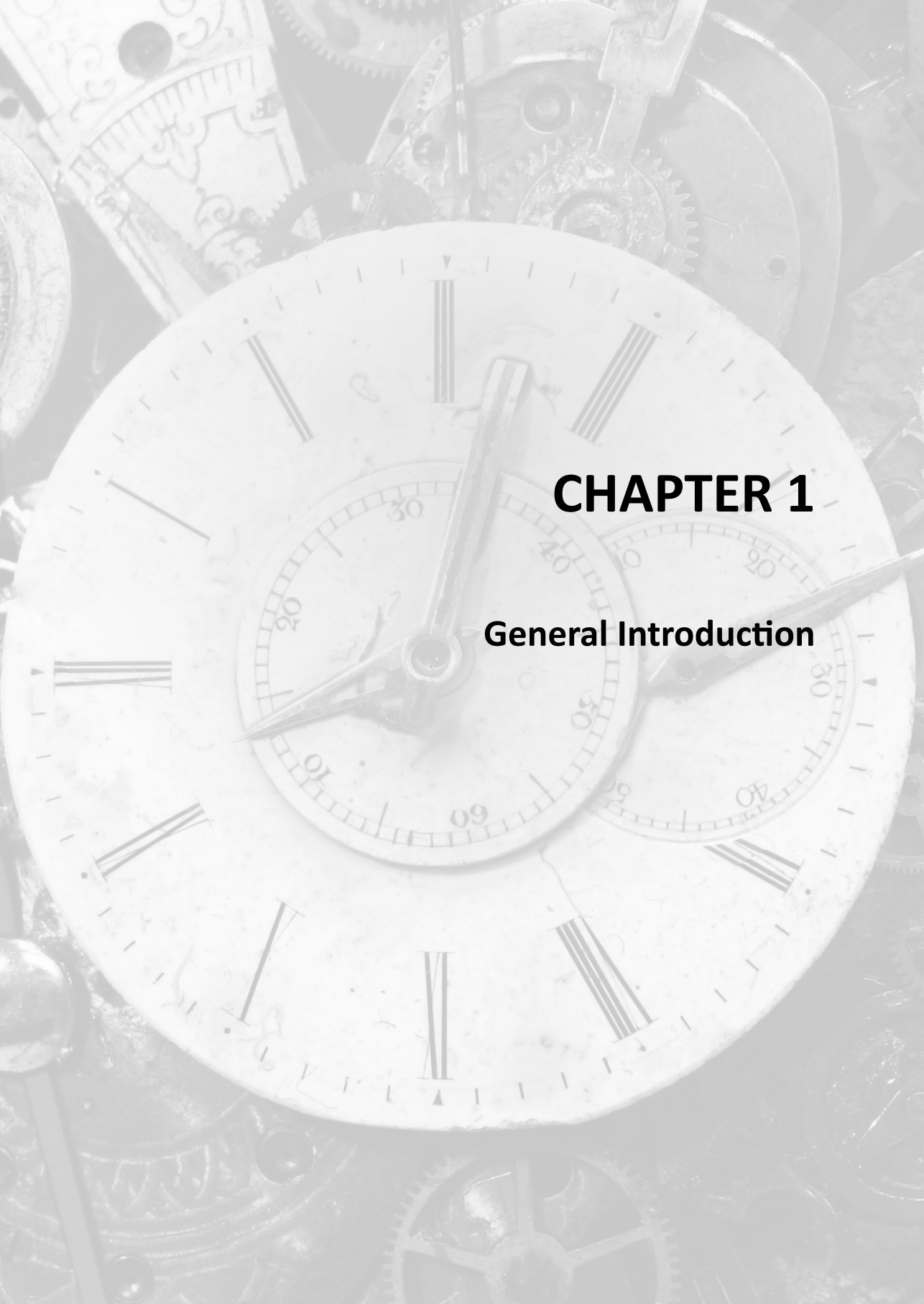
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CHAPTER 1

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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CHAPTER 2

Interacting epidemics? Sleep curtailment, insulin resistance, and obesity

Eliane A. Lucassen,[1,2] Kristina I. Rother,[3] and Giovanni Cizza[4]

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1. Clinical Center, National Institutes of Health, Bethesda, United States.
2. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
3. Section on Pediatric Diabetes and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
4. Section on Neuroendocrinology of Obesity, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.

Abstract

In the last 50 years, the average self-reported sleep duration in the United States has decreased by 1.5–2 hrs in parallel with an increasing prevalence of obesity and diabetes. Epidemiological studies and meta-analyses report a strong relationship between short or disturbed sleep, obesity, and abnormalities in glucose metabolism. This relationship is likely to be bidirectional and causal in nature, but many aspects remain to be elucidated. Sleep and the internal circadian clock influence a host of endocrine parameters. Sleep curtailment in humans alters multiple metabolic pathways, leading to more insulin resistance, possibly decreased energy expenditure, increased appetite, and immunological changes. On the other hand, psychological, endocrine, and anatomical abnormalities in individuals with obesity and/or diabetes can interfere with sleep duration and quality, thus creating a vicious cycle. In this review, we address mechanisms linking sleep with metabolism, highlight the need for studies conducted in real-life settings, and explore therapeutic interventions to improve sleep, with a potential beneficial effect on obesity and its comorbidities.

Introduction

According to the National Health and Nutrition Examination Survey (NHANES) from 2009 to 2010, 33% of adult Americans were overweight (body mass index (BMI) of 25.0–29.9 kg/m^2), and an even larger proportion (36%) were obese (BMI ≥ 30.0 kg/m^2).[1] The report contained good and bad news: for more than a decade, the age-adjusted prevalence of obesity had not changed in women overall, but there were marked ethnic, gender, and socioeconomic differences. Mexican American and Black women and men continued to show a significant rise in the prevalence of obesity. These figures are reflected in important obesity-related complications, such as hypertension, hyperlipidemia, and type 2 diabetes, all leading causes of cardiovascular disease.[2] Type 2 diabetes affects 8.3% of the U.S population (27% of U.S. residents aged 65 yrs and older), whereas 35% of U.S. adults have fasting glucose and glycosylated hemoglobin (HbA1c) levels in the prediabetic range.[3] Thus, it is of great importance to characterize contributing etiological factors in order to effectively counteract the obesity and diabetes epidemics. The exact mechanisms explaining the relationship between short sleep, obesity, and diabetes remain unclear.

Accumulating evidence has pointed to an association between sleep curtailment and obesity,[4,5] type 2 diabetes,[6–9] and possibly mortality (see Ref. 10 for a review). In the last 50 years, self-reported sleep duration in the United States has decreased by 1.5–2 hrs due to lifestyle changes in our society.[11] Worldwide, secular trends in adult sleep duration may be more variable. A recent review article summarizing 12 studies from 15 countries from 1960 to 2000 did not find a consistent decrease in self-reported adult sleep duration: self-reported sleep duration had increased in seven countries, decreased in six, and results were inconsistent for the United States and Sweden.[12] Imprecision in assessing sleep duration, as well as socioeconomic and demographic factors may play a role in this heterogeneity.

In this paper, we will first present the epidemiologic evidence for these relationships; we will then focus on sleep physiology and its role in metabolic functions. Then, we will critically evaluate the studies of sleep manipulation on glucose metabolism and energy expenditure (EE). We will highlight endocrine parameters that may influence these processes, including thyroid hormones, growth hormone (GH), cortisol, sympathovagal balance, and the immune system. We will also delineate the relationships between short sleep and some of the hormones that control appetite, including leptin, ghrelin, orexin, and neuropeptide Y (NPY). In addition, we will present evidence for the reverse effect: of obesity/diabetes on sleep. Finally, we will describe emerging evidence for a role of melatonin in the sleep–metabolism relationship and its therapeutic potential in metabolic disturbances.

Epidemiologic evidence for an association among sleep duration, obesity, and diabetes

A meta-analysis of 45 cross-sectional studies, including 604,509 adults and 30,002 children, confirmed the relationship between short sleep (generally fewer than five hrs per night in

adults and fewer than 10 hrs per night in children) and obesity, and quantified the associated risk (OR 1.55; 1.43–1.68; OR 1.89; 1.46–2.43, for adults and children, respectively).[13] A similar OR of 1.58 (1.26–1.98) was found in children in another meta-analysis.[14] Most of the included studies used actigraphy or self-reported sleep duration. A polysomnography study, not included in these meta-analyses, of 2,700 men above the age of 65 yrs revealed an inverse relationship between slow wave sleep (SWS) duration and BMI or waist circumference.[15] Another study reported that obese adults (BMI 41 ± 1 kg/m^2) without sleep apnea slept 88 min fewer than lean subjects.[16] The relationship between short sleep and BMI was also confirmed by actigraphy in 612 participants (35–50 yrs old).[17]

In principle, causality is best addressed in prospective randomized controlled studies. This ideal approach may not be practical in this case: studies of sleep extension or curtailment cannot be blinded, and participants may not behave according to the allocation group (i.e. control subjects changing sleep duration or, *vice versa*, intervention subjects not changing sleep duration). For these reasons, the existing prospective studies have all been observational in nature. Short sleepers experience greater weight increases over time according to most,[4,18–22] but not all studies.[17,23,24] However, average weight gain was modest and of relative clinical meaning. Short sleepers (fewer than five to six hours) gained 2 kg more in a 6-year study,[19] and 0.4 kg more in another 16-year study.[18] In addition, subjects had a 35% and 31% greater chance of gaining more than 5 kg in 6 yrs, and gaining more than 15 kg in 16 yrs, respectively.[18,19] Thus, there is a large variability among studies in weight gain associated with short sleep for unknown reasons: the magnitude of this effect may be gender dependent or depend on other yet-to-be identified genetic and environmental factors. For example, short sleep may affect metabolism differently in natural "short sleepers" *vs.* chronically sleep-deprived individuals. A large study including 35,247 subjects found that lean men, but not lean women, who slept fewer than five hrs had increased odds of becoming overweight within one year (OR 1.91; 1.36–2.67).[25] In contrast, in a cohort of 3,576 elderly subjects, women, but not men, who slept fewer than five hours, had a higher risk of gaining 5 kg in two yrs (OR 3.41; 1.31–8.69).[20] Interestingly, in this study, BMI was also increased in women sleeping more than eight hrs. A similar U-shaped association has been reported in other studies.[19,25] Collectively, these studies indicate that self-reported short or long sleep is associated with increased BMI. The amount of weight gain over time, however, varied greatly, ranging from less than 1 kg to several kg, and age may be one of the parameters modulating this association.

Many mechanisms may be responsible for the frequently observed insulin resistance in obese individuals, including increased release of free fatty acids, leptin, and TNF-α from adipose tissue.[26] We suggest that short sleep (fewer than five to six hours) may now qualify as an additional clinical factor in the development of insulin resistance and diabetes, as shown in several cross-sectional studies.[27–29] A recent study of 174,542 middle-aged subjects

showed that short sleepers had an OR of 1.46 (1.31–1.63) for developing diabetes after 3–10 yrs of follow-up.[30] A meta-analysis of 10 prospective reported a pooled OR of 1.28 (1.03–1.60) for diabetes in short sleepers.[31] Interestingly, this correlation was only present in men (OR 2.07; 1.16–3.72). Both long sleep (OR 1.48; 1.13–1.96) and decreased sleep efficiency also resulted in greater chances of developing diabetes.[31] Difficulty in falling asleep resulted in a greater incidence of diabetes after 4.2–14.8 yrs of follow up.[32–34] In smaller studies, the association between sleep duration and diabetes is more variable.[7–9,35] In addition, this variability may be real and differ on the basis of ethnic group and socioeconomic status: a study found increased risk of developing diabetes only in Whites and Hispanics who slept fewer than seven hrs.[36] Besides sleep-duration, poor sleep quality relates to insulin resistance. Frequency of nocturnal awakenings, as measured by actigraphy, correlated positively with HbA1c levels in patients with type 2 diabetes.[37] Furthermore, sleep disordered breathing was related to worse blood glucose control.[38] Taken together, there is solid epidemiologic evidence for an interconnection between sleep duration (too short or too long), insulin resistance, and obesity.

Physiological changes of metabolism during normal sleep

Sleep architecture, the structure and pattern of sleep, is best described by a combined approach that uses electroencephalographic, electromyographic, and other criteria (**Fig. 1**).[39] Many metabolic parameters display diurnal rhythms, which are the resultant of both homeostatic and circadian mechanisms. From a homeostatic point of view, sleep pressure increases with sustained wakefulness. Circadian rhythms are endogenously generated in the suprachiasmatic nucleus of the hypothalamus by feedback loops of transcripts of a group of "core clock" genes, including the CLOCK gene.[40,41] Timekeeping signals are passed on from the suprachiasmatic nucleus to oscillators in peripheral tissues also containing core clock genes. Endogenous rhythms, in turn, are synchronized to the outside world by various environmental clues, mostly by light via the retina. During sleep, energy requirements are lower as metabolic needs for processes such as breathing, gut motility, heart rate and muscular activity, are decreased.[42] Indeed, resting EE, as determined by indirect calorimetry, was lower in healthy volunteers during sleep compared to wakefulness.[43]

During sleep glucose levels remain stable or fall only minimally in spite of fasting, mostly because of decreased energy needs.[44] In contrast, if subjects are kept awake in bed while fasting for a comparable number of hours, glucose levels do fall, approximately 15 mg/dl.[45] During the first part of the night, when most slow wave sleep (SWS) occurs, glucose production is diminished (**Fig. 2**).[44] Brain glucose metabolism, as measured by PET, decreases by 30–40%.[46,47] During the second half of the night, glucose consumption increases due to increased REM sleep requiring more energy.[46,47]

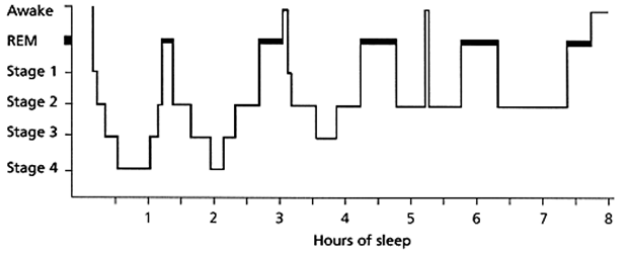


Fig. 1. Representative sleep architecture during eight hrs of uninterrupted sleep (adapted from Ref. 39). Sleep can be divided into rapid eye movement (REM) and nonrapid eye movement (NREM) sleep. On average, adults spend 75–80% in NREM sleep, which can be further subdivided into stages 1 to 4. Stages 3 and 4 are often referred to as slow-wave sleep (SWS) because of the appearance of well-defined waves of 0.5–4.0 Hz. In REM sleep, a phasic and a tonic phase can be distinguished. Skeletal muscles are atonic or hypotonic during REM sleep (except for the diaphragm, extraocular, and sphincter muscles), but bursts of muscle activity can occur during the phasic phase. Furthermore, dreams mostly take place during REM sleep and are more complex during this stage. Eye movement is only present during the phasic phase. Throughout a normal night of sleep, there are three to five sleep cycles, from NREM (stage 1 to 4) to REM, each cycle taking 90–120 minutes. Humans display less SWS and more REM sleep toward the end of the night.

EE and glucose metabolism are modulated by a number of hormones. Thyroid hormones are important determinants of EE; cortisol and GH are powerful modulators of circadian rhythms of glucose metabolism. The sympathovagal system and adiponectin influence both EE and glucose metabolism. Thyroid stimulating hormone (TSH) levels rise at night, mostly due to increased hypothalamic secretion of thyroid releasing hormone (TRH) (**Fig. 2**).[48]

GH secretion is under dual hypothalamic control: somatostatin is inhibitory and growth hormone releasing hormone (GHRH) is stimulatory. Somatostatin and GHRH are secreted in alternation, thus generating the typical pulsatile pattern of GH secretion. GH typically peaks at sleep onset during SWS (**Fig. 2**).[49] GH levels were lower in SWS-deprived individuals[50] and higher in individuals with pharmacologically induced SWS.[51] In addition, REM sleep deprivation did not affect plasma GH levels.[52] Intravenous GH administration decreases SWS, suggesting a modulatory role for GH on SWS.[53,54] The GH peak at sleep onset has insulin lowering effects.55 GHRH injection increased glucose levels at awakening by almost 50%[56] and GH administration rapidly decreased muscular glucose uptake.[55,57]

Cortisol concentrations reach their zenith in the morning, experience a gradual fall during the day, which is briefly interrupted by meals, and have their nadir around 3 am (**Fig. 2**).[48] Cortisol impairs insulin sensitivity with a latency of four to six hrs.[56,58,59] Plasma insulin levels also display a modest diurnal variation with a 10% excursion, a nadir between midnight and 6 am and a peak between noon and 6 pm.[60] Adiponectin is a hormone secreted by the adipocytes in an inverse fashion compared to fat mass.[61] Its role in energy homeostasis is unclear: increases, decreases, or no changes in energy balance are derived from peripheral adiponectin administration in rodents.[62] Adiponectin knockout mice develop insulin resistance and increased TNF-α levels when fed a high fat diet. In humans, plasma adiponectin levels correlated negatively with insulin resistance, and higher levels correlated with future weight gain in 1,063 women.[63,64]

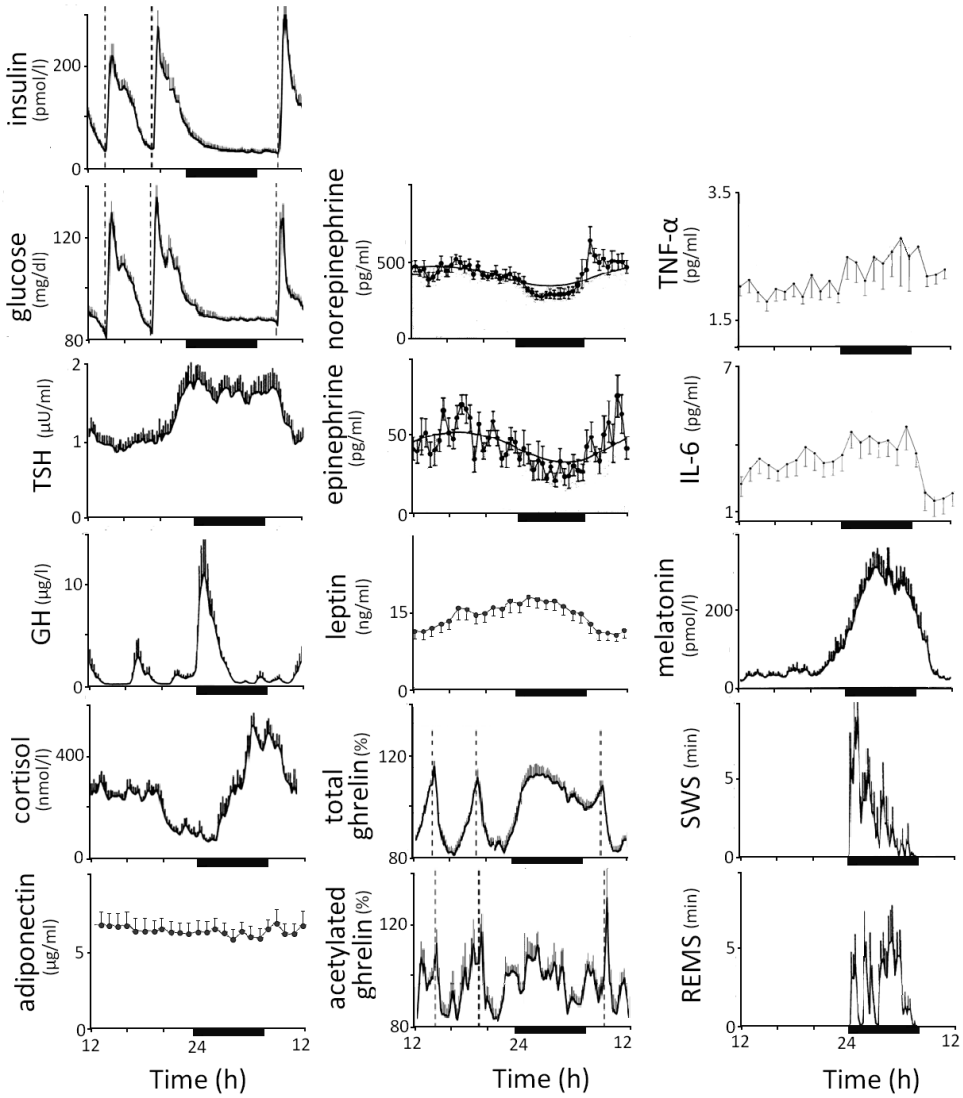


Fig. 2. Mean levels of metabolic parameters and sleep stages in lean young men ($n = 8$ for TSH, cortisol, GH, melatonin, SWS, and REM sleep;[48] $n = 14$ for insulin, glucose, total and acetylated ghrelin;[76] $n = 8$ for catecholamines[73]); in 23 lean women for leptin and adiponectin;[69] and in 25 individuals (13 females, 12 males) for IL-6 and TNF-α.[83] Ghrelin levels are indicated as a percent of mean 24 hrs. values (1027 pg/mL for total ghrelin; 80 pg/mL for acetylated ghrelin); leptin levels are shown as percent change from levels at 08:00. Dark bars below each plot indicate bedtimes. Sleep was monitored polysomnographically during all measurements. Striped bars indicate meal times. Modified with permission from Refs. 48, 69, 73, 76, and 83.

Individuals with type 2 diabetes typically have low adiponectin levels, even when adjusted for body weight.[65] In contrast, patients with type 1 diabetes have higher than expected adiponectin levels: an observation that is poorly understood.[66] Sleep duration was inversely related to serum adiponectin in 109 lean Japanese males.[67] Adiponectin has anti-inflammatory properties: *in vitro* exposure to adiponectin decreases macrophage activation and TNF-α production. Finally, adiponectin displays some diurnal variability, with lower levels at night (**Fig. 2**).[68,69]

During sleep, vagal tone increases.[70] Sympathovagal balance is lowest during REM sleep, when the locus coeruleus, the main brain source of catecholamine biosynthesis, is silent.[71] Plasma norepinephrine (NE) and epinephrine (EPI) are produced by the sympathetic system: EPI is secreted exclusively from the adrenal medulla, whereas NE can also be released from postganglionic sympathetic nerves.[72] In a small study of eight lean males, plasma catecholamines were consistently lower during the night with no differences among sleep stages (**Fig. 2**).[73] Heart rate and mean blood pressure were lower during NREM sleep, compared to during awakenings and REM sleep.[70]

Several hormones involved in appetite regulation display circadian rhythms. Leptin is secreted by the white adipose tissue in a highly pulsatile fashion.[74] Leptin reflects fat stores: its levels increase after meals and during the night and are associated with decreased appetite (**Fig. 2**).[74] Ghrelin, produced in the stomach, stimulates appetite and circulates mainly in a nonacetylated form.[75] Acetylated ghrelin, the active form, is necessary for most of its endocrine actions, including GH release, appetite stimulation, and gastric emptying. Total and acetylated ghrelin levels rise with fasting and during sleep (**Fig. 2**).[76] Leptin and ghrelin exert their effects in the arcuate nucleus of the hypothalamus, through anorexigenic proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) and orexigenic neuropeptide Y/agouti-related protein (NPY/AgRP) neurons. Leptin activates POMC/CART neurons, which induces hunger and inhibits NPY/AgRP neurons,[77,78] which induces satiety. Ghrelin has the opposite effect on the arcuate nucleus, stimulating appetite, and prolonging postprandial glucose responses, while stimulating GH release.[79] Insulin, while peripherally lowering blood glucose and stimulating appetite, inhibits appetite centrally in a leptin-like manner.[78]

Orexin A and B, two neuropeptides released by lateral hypothalamic neurons, stimulate appetite. Orexin neurons are active during waking and quiescent during sleep; consistently, orexin levels in the CSF are maximal at the end of the waking period.[80,81] The orexin system is activated by ghrelin and inhibited by leptin and glucose. Projections from orexin neurons in the lateral hypothalamus activate NPY neurons in the arcuate nucleus.[80] Melatonin is a sleep-promoting hormone that is produced by the pineal gland. Melatonin is exclusively secreted during the dark, as light inhibits its production (**Fig. 2**).

Interleukin 6 (IL-6), interleukin 1β (IL-1β), TNF-α, and C-reactive protein (CRP) are pro-inflammatory factors. IL-6 displays a circadian rhythm, with a first peak around 2 am and a second peak around 5 am (**Fig. 2**).[82,83] IL-1β decreases during the night and reaches its nadir at 8 am in the morning.[82] TNF- α also displays a circadian rhythm, peaking close to the awakening (6 am) and reaching a nadir around 3 pm (**Fig. 2**). IL-6 and TNF-α stimulate secretion of cortisol and, in turn, cortisol inhibits their secretion.[84] Reports on diurnal variability of CRP are few and inconsistent: some report no diurnal variance,[85] whereas others find increased morning levels.[86] In 10 healthy, lean men, white blood cell counts peaked around 11 pm, decreased throughout the night and reached a nadir at 8 am.[82]

The relationships here described between hormone secretion and sleep stages are necessarily descriptive. In theory, as new pharmacological tools aimed at manipulating hormonal status (i.e., selective hormone agonists/antagonists) and/or sleep become available for investigation, we may be able to better understand the numerous causal links interconnecting these phenomena. In summary, many metabolic parameters involved in EE regulation, glucose metabolism, and appetite control display diurnal rhythms, reflecting the different metabolic needs during the sleep and wake states. In the next section, we will report the effect of sleep deprivation on these metabolic parameters.

Effects of sleep restriction on metabolic parameters

The relationship between sleep and metabolism has been extensively studied in human subjects under varying conditions, such as total sleep deprivation,[87–100] partial sleep deprivation,[101–122] and sleep fragmentation (**Table 1**).[123–125] Partial sleep deprivation affects SWS less than other sleep stages. Most of the experiments on the effects of acute sleep deprivation were conducted in healthy, lean volunteers. Food intake and physical activity were often, but not always, strictly controlled (**Table 1**). It is important to note that experimentally induced sleep alterations may not be representative of changes occurring in clinical condition of chronic sleep deprivation. In addition, important differences exist between acute and chronic sleep deprivation. We will describe effects of sleep curtailment on important metabolic parameters that may contribute to insulin resistance and obesity, including effects on appetite and EE (**Fig. 3**).

Table 1: Results of 37 human experimental studies assessing influence of sleep curtailment on metabolism.

Authors, Year [Reference] **Study design** **Energy intake** **Behavioral activity**	**Gender/Sample Size** **Age ± SEM (yrs)** **Mean weight ± SEM** **Prestudy conditions**	**Experimental sleep protocol**	**Techniques used** **Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
Benedict et al., 2011 [97] Randomized cross-over study Controlled, post-intervention *Ad libitum* Bed rest	men (n = 14) 22.6 ± 0.8 23.9 ± 0.5 kg/m^2 6 wks regular bedtimes*	**1**: 1 night of 8h bedtime*** **2**: 1 night of TSD ≥ 4 wks between conditions	Indirect calorimetry pre- and postbreakfast; morning VAS hunger ratings 24 hours, every 1.5–3 hours	↑ postprandial glucose (8%) ↓ RMR (5%) and postprandial (20%) metabolic rate ↑ cortisol (7%)[1] ↑ NE (12%)[1] ~ food intake; ↑ hunger (60%)[1] ↑ morning ghrelin (11%) ~ leptin
Born et al., 1997 [79] Randomized cross-over study Standardized meals Synchronous inpatient activities	men (n = 10) 24.7 (21-29) NA NA	**1:** 1 night of 8h bedtimes***, then 1 night of *ad libitum* sleep*** **2**: 24h TSD *** ≥ 10 days between conditions	LPS-stimulated whole blood and ELISA (TNF-α; IL-1β; IL-6) 51h, every 3h	~ IL-6 ~ IL-1β; ↑ nocturnal whole blood ~ TNF-α ↑ white blood cells (8%)[1]
Brondel et al., 2010 [110] Randomized cross-over study *Ad libitum* Free physical activity**	men (n = 12) 22 ± 3 22.3 ± 1.83 kg/m^2 2 days sleep/diet diaries*	**1:** 1 night of 4h bedtime*** and 1 night free bedtime at home*,** **2**: 1 night of 8h bedtime*** and 1 night of free bedtimes at home*,** Conditions ≥ 5 days apart	Actigraphy; 12 daytime VAS hunger ratings	~ EE; ↑ physical activity (13%) ↑ hunger; ↑ food intake (22%)
Buxton et al., 2010 [111] Consecutive experimental phases Controlled isocaloric (finishing meal required) Controlled sedentary living	men (n = 20) 26.8 ± 5.2 23.3 ± 3.1 kg/m^2 ≥ 5 days of 10hr bedtimes*,**	**1:** 3 nights of 10h bedtimes*** **2:** 7 nights of 5h bedtimes***	ivGTT penultimate day; clamp last day; salivary sampling 15:00-21:00 last 2 days, every hour, 24h urine sampling last 2 days; indirect calorimetry	↑ insulin resistance (20% by ivGTT; 11% by clamp) ~ fasting RMR ↑ salivary cortisol (51%) ↑ urinary NE (18%) and EPI (22%)[1]
Clore et al., 1989 [41] Comparative study Similar evening meal Bed rest	5 females, 12 males (n = 17) 25 ± 1 24.1 ± 0.6 kg/m^2 NA	**1**. Group 1 (n = 11): 1 night of 8h30min bedtime **2**. Group 2 (n = 6): 1 night of TSD	Continuous ^{3}-3H glucose infusion	↓ nocturnal fall in glucose utilization ↓ nocturnal fall in glucose production

Authors, Year [Reference] **Study design** **Energy intake** **Behavioral activity**	**Gender/Sample Size** **Age ± SEM (yrs)** **Mean weight ± SEM** **Prestudy conditions**	**Experimental sleep protocol**	**Techniques used Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
Donga et al., 2010 [112] Randomized cross-over study NA NA	3 females, 4 males (n = 9) 44 ± 7 23.5 ± 0.9 kg/m^2 Type 1 diabetes, HbA1c ≤ 8.5%, 1 week of regular bedtimes[*,**]	**1**: 3 nights of 8h30min bedtimes[***] **2:** 3 nights of 4h bedtimes[***] Conditions ≥ 3 wks apart	Hyperinsulinemic euglycemic clamp	↑ insulin resistance (21%)
Dzaja et al., 2004 [92] Randomized cross-over study 1,800 kcal/24h Bed rest	men (n = 10) 28 ± 3.1 24.0 ± 2.9 kg/m^2 1 week of regular bedtimes[*]	**1**: 1 night of 8h bedtime[***] **2**: 1 night of TSD Conditions ≥ 2 wks apart	24h, every hour	↓ GH peak (71%)[2] ~ mean cortisol levels ↓ nocturnal total ghrelin increase (39%)[2]
Frey et al., 2007 [93] Consecutive phases study Hourly controlled intake Bed rest	9 females, 10 males (n = 19) 28.1 ± 8.6 18.5-24.5 kg/m^2 3wks of regular bedtimes[*,**]	**1:** 3 nights of 8h bedtimes[***] **2:** 1 night of TSD	Saliva 36h, every hour; hsELISA (IL-6; IL-1β; CRP) 40h, every 30min	~ saliva cortisol; ↓ at 13:00 (53%)[2] and 20:00 (17%)[2] ↓ daytime IL-6 ↑ IL-1β ↓ morning hsCRP
Gonzáles-Ortiz et al., 2000 [87] Consecutive experimental phases Controlled isocaloric Free inpatient movement	7 females, 7 males (n = 14) 21.0 ± 2.1 22.1 ± 1.3 kg/m^2 3 days diet diary	**1:** baseline "normal sleep" **2:** 1 night of TSD	Insulin suppression test with octeotride Once, morning	↑ insulin resistance (18%)[1] ~ morning cortisol
Haack et al., 2007 [105] Comparative study Controlled isocaloric Free inpatient movement	6 females, 12 males (n = 18) 27.3 ± 5.8 23.1 ± 3.3 <30 kg/m^2 ≥ 10 days regular bedtimes[*]	(n = 8) **1:** 10 nights of 8h bedtimes[**] (n = 10) **2:** 10 nights of 4h bedtimes[**]	24h urine 24h blood, every 4h (high sensitivity ELISA IL-6 and hsCRP)	~ food intake ↑ IL-6 (62%)[1] ~ TNF-receptor 1 ↑ hsCRP (102%; P = 0.11)[1]
Hursel et al., 2011 [122] Randomized cross-over Controlled isocaloric Sedentary activity	men (n = 15) 23.7 ± 3.5 24.1 ± 1.9 kg/m^2 2 days controlled feeding	**1.** 2 nights of 8h bedtimes[***] **2:** 2 nights of sleep fragmentation hourly 2-min-long alarms[***] Conditions ≥ 2 wks apart	Indirect calorimetry	~ total EE ↑ physical activity (8%)[1] ↑ RQ (3%)[1]
Irwin et al., 2006 [104] Consecutive phases study NA No exercise	13 females, 17 males (n = 30) 37.6 ±9.8 <30 kg/m^2 2 wks regular bedtimes[*]	**1:** 3 nights of 8h bedtimes[***] **2:** 1 night of 4h bedtime[***]	Flow cytometry; real time PCR (n = 10); high-density oligonucleotide array (n = 5) Daytime (08:00-23:00), every 3-4h	↑ IL-6 monocyte expression (344%)[1]; ↑ IL-6 mRNA (250%)[1] ↑ TNF-α monocyte expression (87%)[1]; ↑ TNF-α mRNA (100%)[1]

Authors, Year [Reference] **Study design** **Energy intake** **Behavioral activity**	**Gender/Sample Size** **Age ± SEM (yrs)** **Mean weight ± SEM** **Prestudy conditions**	**Experimental sleep protocol**	**Techniques used** **Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
Jung et al., 2011 [40] Consecutive experimental phases Controlled isocaloric Sedentary	2 females, 5 males (n = 7) 22.4 ± 4.8 22.9 ± 2.4 kg/m^2 1 week 8h sleep*,**; 3 days diet	**1:** 2 nights of 8h bedtimes*** **2:** 40h of TSD	Indirect calorimetry	↑ total EE (7%); ↑ nocturnal EE (32%); ~ daytime EE ~ RQ
Kuhn et al., 1969 [84] Consecutive phases study Balanced diet NA	human subjects (n = 28) 20 – 30 NA NA	**1:** 4-5 days "control period" **2**: 72-126h of TSD **3:** 3 days "control period"	OGTT Once daily	↓ glucose tolerance after 3–4 days ↑ 17-OH corticosteroids on day 2 and 3, then return to baseline ↑ urinary catecholamines
Mullington et al., 2003 [91] Consecutive phases study Controlled isocaloric (finishing not required) Sedentary	men (n = 10) 27.2 26.1 ± 1.9 kg/m^2 1 week of regular bedtimes*,**	**1.** 3 nights 8h bedtimes*** **2**. 3 nights of TSD	120 hours, every 90 min	~ leptin mesor, ↓ leptin circadian amplitude
Nedeltcheva et al., 2009 [106] **Kessler et al., 2010 [113]** Randomized crossover study *Ad libitum* Sedentary	5 females, 6 males (n = 11) 39 ± 5 26.5 ± 1.5 kg/m^2 NA	**1:** 14 nights of 8.5h bedtimes*** **2:** 14 nights of 5.5h bedtimes*** Conditions ≥ 3 months apart	OGTT; ivGTT 24h, every 15-30min	↓ insulin sensitivity (18%)[1] ↓ TSH (7%; especially in females) [1] ↓ free T4 (8%)[1] ~ GH; ↓ during first 4hours of bedtime period in males (31%)[1] ~ cortisol; ↓ and 44min later acrophase (10%)[1]; 71min later nadir ↑ EPI (21%)[1] ~ mean NE; ↑ nocturnal (24%)[1]
Nedeltcheva et al., 2010 [115] Randomized cross-over study Controlled restricted diet (90% RMR) Sedentary	3 females, 7 males (n = 10) 41 ± 5 27.4 ± 2 kg/m^2 NA	**1:** 14 nights of 8.5h bedtimes*** **2:** 14 nights of 5.5h bedtimes*** Conditions ≥ 3 months apart	Indirect calorimetry; VAS hunger rating scale before meal/at 22:30 24h, every hour	~ total energy expenditure; ↓ RMR (8%)[1]; ↑ fasting and postprandial RQ (4%)[1] ~ thyroid hormones ~ GH ~ cortisol ↓ EPI (12%)[1]; ~NE ~ food intake; ↑ hunger ~ total weight loss (3 kg), ↓ fat weight loss (55%), ↑ fat-free weight loss (60%) ↑ mean acylated ghrelin; (7%)[1] ~ leptin

Authors, Year [Reference] Study design **Energy intake Behavioral activity**	**Gender/Sample Size Age ± SEM (yrs) Mean weight ± SEM Prestudy conditions**	**Experimental sleep protocol**	**Techniques used Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
Omisade et al., 2010 [114] Consecutive phases study Controlled Free inpatient movement	women (n = 15) 21.6 ± 2.2 24.5 ± 8 kg/m^2 1 week regular bedtimes and diet[*,**]	**1:** 1 night of 10h bedtime[**] **2:** 1 night of 3h bedtime[**]	VAS hunger ratings; salivary cortisol daytime, every 1-2h; salivary leptin morning and afternoon	↓ median morning cortisol (19%)[1]; slower decline; ↑ afternoon/evening (44%)[1] ~ hunger ↑ morning leptin (8%)[1]
Parker et al., 1987 [85] Consecutive phases study Controlled diet Sedentary	men (n = 4) 21-30 NA NA	**1:** 1 night of 8h bedtime[***] **2**: 2 nights of TSD	72h, every 30min	↑ TSH; ↑ amplitude, longer peak, later nadir/ acrophase
Pejovic et al., 2010 [96] Consecutive phases study *Ad libitum* Ambulatory	11 females, 10 males (n = 21) 24.1 ± 3.1 24.1 ± 2.6 kg/m^2 2 wks regular bedtimes[**]	**1:** 4 nights of 8h bedtimes[***] **2:** 1 night of TSD, half 2h afternoon nap	Hunger scale (0-10) 24h, every 30 min	~ cortisol ~ adiponectin ~ hunger ↑ leptin (14%)[1]
Schmid et al., 2007 [94] Randomized cross-over study None Bed rest	men (n = 10) 25.3 ± 1.4 23.8 ± 0.5 kg/m^2 Light dinner before arrival in the lab (21:00)	**1**: 1 night of 8h bedtime[***] **2**: 1 night of TSD Conditions ≥ 2 wks apart	Hypoglycemic clamp Two morning samples	↓ glucagon levels (16%)[1]; ↑ response to hypoglycemia ↓ cortisol levels (16%)[1]; ~ ACTH ~ catecholamines ↑ hunger
Schmid et al., 2008 [95] Randomized cross-over study None Bed rest	men (n = 9) 24.2 ± 1.0 23.8 ± 0.6 kg/m^2 NA	**1**: 1 night of 7h30min bedtime[***] **2**: 1 night of 5h bedtime[***] **3:** 1 night of TSD Conditions ≥ 2 wks apart	Hunger graded 0-9 Two morning samples	↑ hunger (129%)[1] ~ leptin ↑ total ghrelin (22%)
Schmid et al., 2009 [107] **Schmid et al., 2011 [116]** Randomized cross-over study *Ad libitum* Free outpatient movement	men (n = 15) 27.1 ± 1.3 22.9 ± 0.3 kg/m^2 4 wks regular bedtimes[*]	**1:** 2 nights of 8h bedtimes[***] **2:** 2 nights of 4h bedtimes[***] Conditions ≥ 6 wks apart	Actigraphy, buffet on Day 2, ELISA (IL-6) 08:00-23:00 (every hour)	↑ insulin (40%) and glucose (11%) peak response to breakfast ↓ physical activity (13%)[1] ~ cortisol; ~ ACTH ~ total energy intake; ↑ fat intake (23%)[1] ~ hunger; ~ intake ~ leptin; ~ ghrelin ~ IL-6
Schmid et al., 2009 [108] Randomized cross-over study None Bed rest	men (n = 10) 25.3 ± 1.4 23.8 ± 0.5 kg/m^2 2 wks regular bedtimes[*]	**1**: 1 night of 7h bedtime[***] **2**: 1 night of 4.5h bedtime[***] Conditions ≥ 2 wks apart	Hypoglycemic clamp Two morning samples	~ insulin resistance ↓ glucagon (8%)[2] ~ GH ↓ ACTH (44%)[1]; ↓ cortisol (44%)[1] ~ EPI; ~ NE

Authors, Year [Reference] **Study design** **Energy intake** **Behavioral activity**	**Gender/Sample Size** **Age ± SEM (yrs)** **Mean weight ± SEM** **Prestudy conditions**	**Experimental sleep protocol**	**Techniques used** **Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
Shearer et al., 2001 [90] Comparative study Isocaloric controlled No exercise	men (*n* = 42) 28.7 (21-47) NA 2 wks regular bedtimes**	(*n* = 21) **1:** 3 nights of 8h bedtimes, then 4 nights of 2h bedtimes (*n* = 21) **2:** 3 nights of 8h bedtimes, 4 nights of TSD	5 days, every 6h (ELISA)	No changes in condition **1**; in condition **2**: ↑ IL-6 ~ TNF-α ↑ sTNF-α receptor I ~ sTNF-α receptor II
Simpson et al., 2010a [117] Consecutive phases study *Ad libitum* Free inpatient movement	67 females, 69 males (*n* = 136) 30.4, 22-45 24.7, 17.7-32.6 kg/m^2 1 week regular bedtimes*,**	**1:** 2 nights of 10h bedtimes **2:** 5 nights of 4h bedtimes	Once, morning (10:30-12:00)	↑ leptin (33%)[1]
Simpson et al., 2010b [118] Consecutive phases study *Ad libitum* Free inpatient movement	33 females, 41 males (*n* = 74) 29.9, 22-45 24.6, 17.7-33.1 kg/m^2 1 week regular bedtimes*,**	**1:** 2 nights of 10h bedtimes*** **2:** 5 nights of 4h bedtimes***	Once, morning (10:30-12:00)	~ adiponectin in men; ↑ in African American women; ↓ in Caucasian women
Spiegel et al., 1999 [98] **Spiegel et al., 2000 [99]** **Spiegel et al., 2003 [100]** **Spiegel et al., 2004a [101]** Consecutive experimental phases Controlled Free outpatient movement	men (*n* = 11) 22 ± 1 23.4 ± 0.5 kg/m^2 ≥ 1 week of 8h bedtimes, diet**,***	**1:** 6 nights of 4h bedtimes*** **2:** 6 nights of 12h bedtimes***	ivGTT; 24h heart rate variability; salivary sampling 15:00-bedtime, every 30min; blood sampling 24h, every 30min	↓ glucose clearance (30%) ↓ TSH (26%), ↑fT4 (7%)[1] ~ GH, different profile ↑ afternoon saliva/plasma cortisol (25%)[1] ↑ sympathovagal balance (17%)[1] ↓ leptin (19%); ↓ and 2.5h earlier acrophase (26%); ↓ amplitude (20%) ↓ melatonin; later onset; ↓ acrophase
Spiegel et al., 2004b [102] Randomized cross-over study Controlled Free outpatient movement	men (*n* = 12) 22 ± 2 23.6 ± 2.0 ≥ 1 week of 8h bedtimes	**1:** 2 nights of 10h bedtimes*** **2:** 2 nights of 4h bedtimes*** Conditions ≥ 6 wks apart	VAS hunger rating scale 08:00-21:00, every 20min	↑ hunger (23%; 33-45% for sweets/salty foods) ↓ leptin (18%) ↑ ghrelin (28%)
Stamatakis et al., 2010 [121] Consecutive phases *Ad libitum* NA	2 females, 9 males (*n* = 11) 23.2 (18 – 29) 24.3 ± 0.9 kg/m^2 3 days diet and >7h bedtimes*,**	**1:** 1 night of unfragmented sleep*** **2:** 2 nights of fragmented sleep (~ 31.4 auditory/ mechanical stimuli per h)***	ivGTT; heart rate variability; enzyme-linked immunosorbent assay techniques (IL-6; hsCRP) 2 morning samples (08:00 and 16:00)	↓ insulin sensitivity (25%) ↑ morning cortisol (13%) ~ adiponectin ↑ sympathetic tone (17%) ~ leptin ~ Il-6; ~hsCRP

Authors, Year [Reference] **Study design** **Energy intake** **Behavioral activity**	**Gender/Sample Size** **Age ± SEM (yrs)** **Mean weight ± SEM** **Prestudy conditions**	**Experimental sleep protocol**	**Techniques used** **Blood sampling (time, frequency)**	**Main findings (sleep deprived *vs.* longer sleep)**
St-Onge et al., 2011 [119] Randomized cross-over study Controlled isocaloric, self-selected on Day 5 Free inpatient movement	15 females; 15 males (n = 30) 33.9 ± 4.3; 36.6 ± 5.6 23.0 ± 1.1; 24.1 ± 1.1 kg/m^2 2 wks regular bedtimes*,**	**1:** 5 nights of 9h bedtimes*** **2:** 5 nights of 4h bedtimes*** Conditions ≥ 4 wks apart	Double labeled water (EE); indirect calorimetry (RMR); actigraphy; VAS hunger scales	~ RMR; ~ EE; ~ physical activity ~ hunger; ↑ food intake (12%)[1]; especially ↑ saturated fat intake in females (62%)
Tasali et al., 2008 [120] Randomized cross-over study Controlled Sedentary activities	4 females, 5 males (n = 9) 20 – 31 64.2 kg; 19-24 kg/m^2 NA 1 week regular bedtimes**	**1:** 2 nights of undisturbed sleep*** **2:** 3 nights of disturbed sleep by acoustic stimuli during SWS (~33 micro-arousals/h)*** Conditions ≥ 4 wks apart	ivGTT; heart rate variability 24h, every 20min	↓ insulin sensivitity (25%) ~ cortisol; ~ daytime; ~ nocturnal ↑ sympathovagal balance (14%)
Thomas et al., 2000 [88] Consecutive experimental phases NA NA	men (n = 17) 24.7 ± 2.8 NA ≥ 7 days of 7h45min bedtimes**	**1:** 3 nights of 7h45min bedtimes*** **2:** 85h of TSD (only results from 24h shown)	18-FDG and PET, every 24h	↓ global cerebral glucose metabolic rate (8%), especially the thalamus, prefrontal and posterior parietal cortices
Van Cauter et al., 1991 [42] Consecutive phases study Only breakfast on study day Bed rest	men (n = 8) 22 - 27 22.7 ± 0.7 kg/m^2 1 week 23:00-07:00 bedtimes*	**1**: 2 nights of 8h bedtimes*** **2**: 28h of TSD	Continuous glucose infusion during phase 2 24 hours, every 30 min	↓ nocturnal GH pulses (70%)[1] ~ cortisol, ↓ acrophase (4%)[1]; 1h earlier nadir
VanHelder et al., 1993 [86] Consecutive phases study Isocaloric controlled Sedentary and exercise	men (n = 10) 22 ± 3 74.5 ± 11.8 kg 1 night of 7h bedtimes***	60h of TSD followed by 7h of recovery sleep*** **1**: sedentary activities **2**: daily exercise Conditions ≥ 10 days apart	OGTT at 10h, 60h and after recovery sleep	↑ insulin response (21% in group 1)
Van Leeuwen et al., 2009 [109] Consecutive phases study Isocaloric controlled Free outpatient movement	men (n = 13) 23.1 ± 2.5 NA 2 wks regular bedtimes*	**1:** 2 nights of 8h bedtimes*** **2:** 5 nights of 4h bedtimes***	Flow cytometry, real time PCR (1 morning sample), salivary cortisol (10 times a day)	~ cortisol ↑ IL-6 (63%) ↑ IL-1β (37%) ~ TNF-α ↑ hsCRP (45%)

Authors, Year [Reference] Study design Energy intake Behavioral activity	Gender/Sample Size Age ± SEM (yrs) Mean weight ± SEM Prestudy conditions	Experimental sleep protocol	Techniques used Blood sampling (time, frequency)	Main findings (sleep deprived *vs.* longer sleep)
Vgontas et al., 2004 [103] Consecutive phases study Usual diet Free outpatient movement (sedentary during sampling)	13 females, 12 males (n = 25) 25.2 ± 3.8 23.8 ± 2.3 kg/m^2 2 wks regular bedtimes**	**1:** 4 nights of 8h bedtime*** **2:** 8 nights of 6h bedtimes***	24h, every 30min (ELISA TNF-α and IL-6)	~ cortisol; ↓ and 2h earlier peak ↑ IL-6 ↑ TNF-α in males; ~ in females

Sleep curtailment was verified by *questionnaires/diaries **by actigraphy or ***by polysomnography. Percentage of change in metabolic parameters was given in the article or calculated[1,2]: from [1]absolute levels in the text/tables or from [2]graph values of the original article. Metabolic measurements are in plasma/serum unless otherwise specified. Bed rest was always required during the sleep deprived state. *Abbreviations:* ~ similar; TSD: total sleep deprivation; VAS: visual analogue scale; RMR: resting metabolic rate; IL-6: interleukin 6; IL-1β: interleukin 1β; TNF-α: tumor necrosis factor-α; EE: energy expenditure; ivGTT: intravenous glucose tolerance test; RQ: respiratory quotient; OGTT: oral glucose tolerance test; REM: rapid-eye movement; GH: growth hormone; ACTH: adrenocorticotropic hormone; FDG: fludeoxyglucose; PET: position emission tomography.

Effects on glucose metabolism

In the course of the night, glucose supply to the brain must remain adequate in the setting of slightly declining plasma glucose levels. Infusion of [3H3]-labeled glucose in healthy, lean subjects revealed decreased glucose usage during the night, whereas in subjects kept awake glucose usage was increased (**Table 1**).[44] Total and partial sleep deprivation reduced glucose tolerance in lean individuals. This has been documented by intravenous glucose tolerance tests (ivGTT),[101,109,114,123,124] oral glucose tolerance tests (OGTT),[87,89,109] insulin suppression tests with octreotide,[90] as well as hypoglycemic and euglycemic hypoinsulinemic clamps (**Table 1**).[97,114] Impaired glucose metabolism was not only shown in healthy study participants, but also in individuals with type 1 diabetes: after a four-hr night in adults with type 1 diabetes, glucose infusion rate decreased by 21%, as assessed by hyperinsulinemic euglycemic clamp (**Table 1**).[115] In addition to altered peripheral glucose metabolism during total sleep deprivation, cerebral glucose uptake decreased, especially in the prefrontal and posterior parietal cortex and in the thalamus of healthy volunteers (**Table 1**).[91] Interestingly, most studies found no compensatory rise in plasma insulin secondary to increased insulin resistance after short sleep,[109,114] fragmented sleep,[123] or total sleep deprivation (**Table 1**).[90] However, increased insulin secretion in response to ivGTTs was found after 60 hrs of sleep deprivation[89] and two nights of sleep fragmentation.[124] Thus, almost all studies find decreased glucose tolerance and a dampened compensatory insulin response after sleep curtailment.

Effects on EE

Two groups found that one night of total sleep deprivation increased EE during the night, whereas the following day, resting and postprandial EE were reduced by 5% and 20%, respectively, overall leading to a positive energy balance (**Fig. 3** and **Table 1**).[43,100] Similarly, Schmid et al. reported that volunteers had decreased spontaneous physical activity after a night of short sleep compared to a night with restful sleep (**Table 1**).[110] Fragmenting sleep for two consecutive nights lead to an increase in the respiratory quotient (RQ), indicating a shift from fat toward carbohydrate oxidation, without affecting total EE (**Table 1**).[125] High RQ predicts weight gain and insulin resistance.[126] Two weeks of partial sleep deprivation (5.5 hrs) decreased resting metabolic rate and increased RQ in a randomized, cross-over study (**Table 1**).[109] In contrast, subjects with chronic insomnia had increased metabolic rate.[127] We have recently shown that poor sleep quality was associated with higher REE, a higher RQ, and an activation of the stress system in obese subjects with short sleep duration.[128] In summary, EE may be differentially affected by chronic *vs.* acute sleep curtailment and may also depend on differences in reasons of sleep curtailment (experimentally induced short sleep *vs.* chronic insomnia).

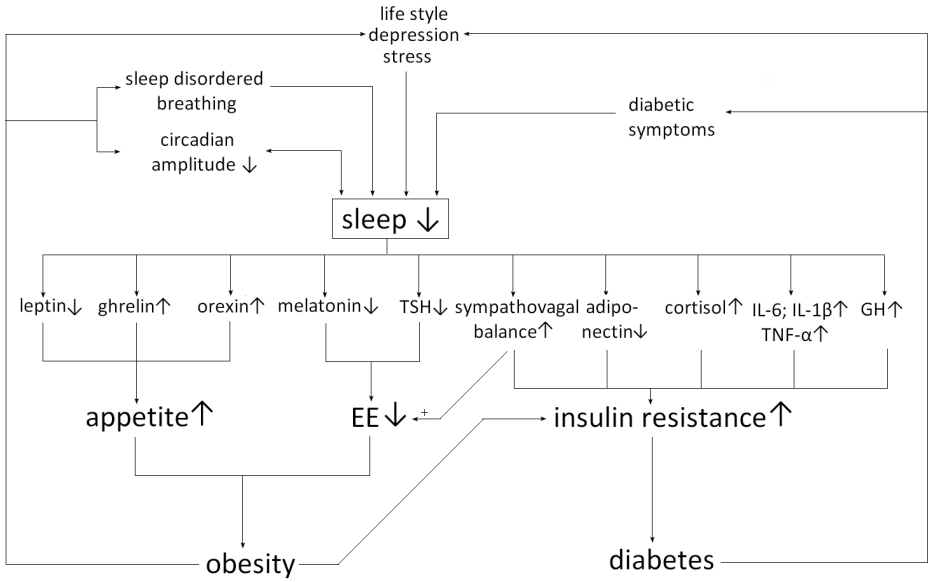


Fig. 3. A simplified schematic representation of putative pathways of sleep curtailment leading to obesity and diabetes, via endocrine mechanisms that stimulate appetite, decrease energy expenditure, and increase insulin resistance. The direction of change (an increase or decrease) of each mechanism due to sleep loss is displayed; increased sympathovagal balance could stimulate EE, depicted by +, while a decreased EE would contribute to obesity. For readability, the relationships between decreases in leptin levels associated with IL-6 and TNF-α production are not displayed. Cortisol and proinflammatory cytokines display a positive bidirectional relationship. Increased insulin levels stimulate orexin secretion and orexic arcuate neurons, while decreasing the activity of anorexic arcuate neurons, leading to a further increase in appetite. Adiponectin influences energy homeostasis, possibly via modulating appetite through arcuate neurons and modulating EE.

Additional endocrine mechanisms linking sleep, glucose metabolism, and energy expenditure

As mentioned above, thyroid hormones, GH, cortisol, adipokines (e.g., adiponectin), and sympathovagal balance modulate glucose metabolism and energy homeostasis (**Fig. 3**). Acute total sleep deprivation increased TSH and free T4 levels,[88] whereas partial sleep deprivation of 5.5 hrs nights for two wks or six 4-hr nights decreased TSH and T4 levels, suggesting a dose-related effect (**Table 1**).[104,116] Decreased peripheral thyroid hormone levels are likely secondary to suppression of the nocturnal TSH peak, possibly secondary to decreased drive from TRH neurons in the PVN.[116] Sleep-deprived rodents show reduced levels of hypothalamic TRH mRNA in the PVN of the hypothalamus.[129] Of note, a different stressor such as acute immobilization also decreases TRH mRNA hypothalamic levels and plasma TSH levels in rodents.[130]

Insulin resistance can result from increases in insulin counter-regulatory hormones, including GH and cortisol (**Fig. 3**). One night of total sleep deprivation markedly decreased the GH peak that usually occurs around sleep onset (**Table 1**).[45,95] Partial sleep deprivation did not affect 24-hr levels of GH in lean or obese individuals,[101,109,118] but six 4-hr nights changed GH nocturnal secretory patterns: sleep deprivation was associated with a biphasic pattern compared to the usual single large GH peak at sleep onset.[102] This extended exposure to GH may decrease glucose uptake in muscles, thus contributing to insulin resistance. However, the threshold of GH levels contributing to insulin resistance is unknown. In contrast to the previous study, decreased GH levels during the first four hrs of sleep were reported after a two-week partial sleep-deprivation period, in association with more SWS.[109,118] GH is usually secreted during SWS, thus this unexpected finding may be due to a dissociation of GH secretion from SWS. In summary, the effects of sleep deprivation on GH plasma levels are variable.

Partial and total sleep deprivation resulted in elevated salivary and plasma cortisol the following afternoon and evening (**Table 1**),[100,101,109,114,117] while morning cortisol levels may be unchanged or even decreased.[90,97,117] As awakening is usually associated with a cortisol surge, this finding probably reflects the lack of the awakening response. In addition, a delay and/or a reduction in the acrophase of cortisol have been reported after partial and total sleep deprivation.[95,102,109] In a sleep-deprived state, cortisol levels decreased more slowly after reaching their acrophase, possibly indicating decreased sensitivity to the negative feedback effects on the hypothalamic–pituitary–adrenal axis. Mean 24-hr levels of plasma cortisol often remain unchanged by sleep deprivation,[43,95,96,99,106,109,123] but the circadian cortisol-pattern changes, as described above.

Adiponectin levels were similar after partial and total sleep deprivation and sleep fragmentation (**Table 1**).[99,121,124] However, subgroup analysis revealed that effects of sleep restriction on adiponectin levels may be gender and race dependent: adiponectin decreased

in Caucasian women and increased in African American women, while adiponectin levels in males did not change after short sleep (**Table 1**).[121] Besides influencing energy homeostasis, decreased adiponectin levels worsen insulin resistance.

Sympathovagal balance, defined as the ratio between the activity of the sympathetic and the parasympathic nervous system, can be indirectly assessed with heart rate variability analysis. Since the activity of this system is very diverse, on the basis of the different anatomic branches, it is unclear to which extent perturbations in heart-rate variability may be predictive of changes in basal metabolic rate and insulin sensitivity. Furthermore, heart rate, EPI and NE levels are markers of sympathetic activity. Acute sleep deprivation is associated with increased sympathetic activity, decreased parasympathetic tone and therefore, with increased sympathovagal balance (**Table 1**).[101,109,123,124] Total EE is usually decreased in acute sleep deprivation; thus, other mechanisms, such as decreased thyroid hormones or adiponectin levels, must prevail over increased sympathovagal tone (**Fig. 3**). Increased sympathovagal balance also adds to insulin resistance (**Fig. 3**).[131] Furthermore, activation of the sympathetic nervous system and inhibition of the parasympathetic nerves decreases insulin secretion; thus, changes in the autonomic nervous system could explain the absence of a compensatory insulin response after sleep curtailment.[132]

In summary, sleep loss impairs glucose metabolism and may decrease EE. This is mediated by multiple factors, such as decreased thyroid hormones and, possibly, adiponectin levels; increased sympathovagal balance; and altered patterns of cortisol secretion, while the role of GH is still unclear (**Fig. 3**).

Effects on appetite

It is not clear to what extent obesity in subjects with short sleep is caused by increased energy intake *vs.* decreased EE (**Fig. 3** and **Table 1**).[133] If food is provided ad libitum, partial or total sleep deprivation may not change hunger ratings, while likely increasing food intake.[99,117,120] On the other hand, when food intake is controlled, sleep deprivation usually increases appetite. Self-reported sleep quality was inversely related to appetite in 53 first degree, normal weight relatives of subjects with diabetes.[134] After two 4-hr nights, there was a 24% increase in daytime appetite ratings;[105] in addition, sleep deprivation specifically increased appetite for sweet and salty foods. The effects of sleep deprivation on appetite may not be immediate: one 4-hr night of sleep deprivation was associated with increases in food intake 36 hrs later, while this parameter was unaffected immediately following sleep deprivation.[113] In addition, the amount of sleep deprivation needed to increase hunger ratings may be variable. One night of total, but not partial, sleep deprivation, significantly increased hunger ratings in normal weight males.[98,113] No differences in total food intake were observed after two 4-hr, 15 min *vs.* 8-hr, 15-min nights, but subjects ate relatively more fat when sleeping less.[119] In a randomized, cross-over, five-day study of 4 hrs *vs.* 9 hrs

of sleep, with a controlled diet on days 1–4 *vs.* an ad libitum diet on day five women, but not men, exhibited increased total caloric intake and a preference for fatty foods, especially if saturated, suggesting that women may be more susceptible to the orexic effects of sleep deprivation than men.[122]

Chronotype, the individual attribute determining morning or evening preference, modulates metabolism. Obese subjects carrying the CLOCK 3111TC/CC polymorphism, a variant of one of the core clock genes associated with being an "owl" or an evening person, sleep 20 minutes less.[135] In addition, these subjects had a higher intake of transfatty acids and proteins, and consumed relatively later in the day, while having the same total energy intake of noncarriers. Subjects carrying the CLOCK 3111TC/CC genotype are also more resistant to weight loss: they lost about 2 kg less while on a hypocaloric diet for 30 wks.[135]

Leptin and ghrelin inhibit and stimulate appetite, respectively (**Fig. 3**). Following partial sleep deprivation, mean daytime values of leptin were approximately 18% lower compared to the rested state.[104,105] In addition, the circadian amplitude of leptin was dampened and the acrophase was decreased and phase advanced by two hrs. A decreased circadian amplitude was also observed after three nights of total sleep deprivation.[94] In a large cohort study of 1,024 subjects, shorter habitual sleep duration as assessed by sleep diaries related to lower morning values of leptin.[5] Some studies found no association between short sleep and leptin levels.[94,98,100,119,136] On the contrary, morning leptin levels were found to be increased after five nights of four hours,[120] after one night of three hours[117] and after one night of total sleep deprivation.[99] Short sleep duration, as assessed by one night of polysomnography in 561 subjects, also related to higher morning leptin levels.[137] It is difficult to explain the inconsistent results of these different studies. Of note, studies that found decreased leptin after short sleep controlled food intake, whereas when sleep-deprived subjects were allowed to eat ad libitum, leptin levels were unchanged or even increased.

In obese subjects, leptin levels are elevated but several of its actions are impaired, a concept referred to as leptin resistance. In a cross-over study design, no association among habitual sleep duration, measured actigraphically, and morning leptin levels was observed in obese subjects.[138] Mean 24-hr leptin levels in obese subjects on a controlled diet were also unchanged after 14 nights of 5.5 hrs *vs.* 8.5 hrs.[118]

The physiological increase in total ghrelin levels in the early night was blunted when individuals were kept awake.[95] A similar phenomenon is reported in chronic insomniacs.[135] However, morning total ghrelin levels were increased after one night of sleep deprivation[95,98] and in individuals with shorter habitual sleep durations.[5] Mean daytime total ghrelin levels were higher after two nights of 4-hr sleep.[105] CLOCK 3111TC/CC carriers, who display an evening preference and sleep 20 min shorter, also had increased morning ghrelin levels.[134] Mean 24-hr levels of the metabolically active acetylated ghrelin were higher in obese subjects after two wks of 5.5-hr nights.[118] Overall, sleep curtailment is consistently associated with higher daytime ghrelin.

The effect of sleep deprivation on the orexin system in normal subjects is not well studied. However, it is known that patients with narcolepsy have abnormally low orexin-A CSF levels, which correlate both with body weight and sleep abnormality.[139] Total sleep deprivation increased orexin levels in the CSF of squirrel monkeys, and increased cFos expression in orexin neurons in rats.[81,140] It is challenging to assess the activity of the orexin system in humans. Central determination requires collection of CSF, an invasive technique. Determination of orexin in plasma is hampered by the lack of adequate assays, in terms of sensitivity and specificity. Hyperactivity of the orexin system could contribute to increased food intake during sleep deprivation. In summary, appetite and food intake are often increased following sleep curtailment, possibly via decreased leptin, increased ghrelin and a hyperactive orexin system (**Fig. 3**).

Effects on the immune system

Enhanced levels of circulating leucocytes, proinflammatory cytokines (e.g., IL-6, TNF-α, IL-1β) and CRP have been associated with an increased risk of cardiovascular disease and type 2 diabetes in long-term human and experimental studies.[141–144] Increased plasma levels of IL-6 were reported after partial and total sleep deprivation in most cases,[93,106,107,108,112] although two studies only reported a loss of circadian rhythms (**Table 1**).[82,96] IL-1β was elevated after partial,[106,112] and total sleep deprivation,[96] but one study did not find any changes after total sleep deprivation (**Table 1**).[82] TNF-α levels are most often,[82,93,112] but not always,[107] unchanged after partial sleep restriction. One study only found increases in men, but not in women, after partial sleep deprivation (**Table 1**).[106] Another study reported higher levels of soluble TNF-α receptor I, through which TNF-α exerts its sleep promoting function.[93] Effects of sleep curtailment on CRP levels are inconsistent: they have been reported lower,[96] unchanged,[124] or higher.[108,112] Prolonged total sleep deprivation has been associated with leucocytosis (**Table 1**).[82] Thus, differences in study outcomes may be influenced by individual or gender differences. In addition, sensitivity and specificity of commercial cytokine assays are highly variable. Highly sensitive analytical methods, such as mass spectrometry, may be needed for accurate cytokine measurements.[145] We conclude that short sleep promotes a proinflammatory state, which, in turn, exerts its negative consequences on insulin resistance.

The other side of the coin: metabolic dysfunction influencing sleep

Most of the studies of sleep deprivation have been performed in healthy, lean subjects and therefore could not address the effect of sleep deprivation on subjects with obesity and diabetes. Obesity and diabetes are often associated with increased stress or differences in life style that could negatively influence sleep (**Fig. 3**).[146] Depression and obesity may occur together as well: obesity may negatively affect mood and both pharmacologic antidepressant therapy and major depression itself are associated with weight gain.[147] We

recently reported that obese subjects often suffer from sleep-disordered breathing, which induces frequent microawakenings and loss of SWS.[148] Similar effects of BMI on sleep have also been observed in youth. Decreased total sleep time and less sleep efficiency were observed in severely obese adolescents, of which 74% displayed sleep apnea (16.5 yrs; BMI 60.3 ± 2.1 kg/m^2).[149] Changes in sleep architecture in obesity have been reported: severely obese adolescents (12–15 yrs; BMI 50.9; 39.8–63.0 kg/m^2, apnea index 14 episodes/hr) have a decrease in REM sleep.[150]

Symptoms associated with diabetes, such as thirst, nocturia, extreme glucose excursions, and mood alterations, may also interfere with sleep, independent of obesity. To this point, adults with type 1 diabetes, who are typically not obese, report a poorer sleep quality than their matched controls without diabetes.[151] These awakenings were associated with a rapid decline of glucose levels, but not with the absolute values of hypoglycemia.[152] Furthermore, studies in animals suggest that diet composition may directly influence sleep: mice fed a high-fat diet display an attenuated rhythm in the circadian expression of core clock genes.[40] Of note, augmented amplitude of the peripheral and central circadian rhythms favors wakefulness during the day and sleep at night, whereas an attenuated circadian amplitude disrupts the sleep–wake cycle.[153] Likewise, studies in rodents demonstrate that physical activity at the congruent time (i.e. nocturnal for mice) increases the central circadian amplitude, suggesting that active behavior during the day and sleep at night in humans may be beneficial for high-amplitude circadian rhythms.[154] Thus, sleep and circadian rhythm are bidirectionally connected (**Fig. 3**). Leptin influences sleep architecture in normal fed rodents, increasing SWS by 13% and decreasing REM by 30%.[155] Furthermore, ob/ob mice that lack leptin and db/db mice that lack the leptin receptor, both display sleep disturbances that are reverted by leptin replacement.

In summary, metabolic dysfunction, altered hormone levels, and sleep abnormalities probably all influence each other.[156,157] In summary, sleep deprivation and obesity may potentiate each other in a vicious reverberating circuit; short sleep may induce weight gain, via the mechanisms outlined in this paper, whereas obesity, in turn, via sleep apnea and other symptoms, may disrupt sleep. Observational studies, especially if cross-sectional, may not be well suited to disentangle these aggregate, causative factors. This underlies the need for behavioral, life-style intervention studies.

Possible therapeutic role of melatonin

In nonequatorial areas, the duration, intensity, and spectral quality of natural light vary across seasons.[158] For example, during a summer in England, there was relatively more blue light *vs.* green and red light exposure, in addition to increased light intensity and duration.[158] These are important signals for seasonal rhythms, transmitted to the body via the duration of nocturnal melatonin secretion. As a consequence of artificial lighting and

other environmental modifications, seasonality is greatly attenuated in modern humans. Furthermore, because sleep is usually the only time during which humans do not experience light exposure, shorter sleep duration will result in longer light exposure. Light suppresses the pineal production of melatonin in a dose related fashion. White artificial light greater than 200 lux completely suppressed nocturnal plasma melatonin production, dim room light (106 lux) reduced melatonin by 88% and light intensities lower than 80 lux did not affect melatonin levels.[159] As the night progresses, the intensity of continuous artificial light needed to suppress circulating melatonin increases.[160] Of note, light of shorter wavelength, such as the light that is typically emitted by electronic devices such as televisions and computers, induces relatively more melatonin suppression and likely leads to greater sleep disruption.[160]

Urinary 24-hr melatonin concentrations decreased by more than 50% when subjects slept five *vs.* eight hours, while being continuously exposed to white light (700 lux) during wake hours.[161] Diminished levels of melatonin were likely due to three additional hrs of light exposure. Likewise, mean 24-hr plasma melatonin concentrations were decreased after 6 nights of 4 hrs *vs.* 12 hrs bedtimes.[103] The onset of melatonin secretion was delayed, due to four hrs longer light exposure before bedtime (300 lux) and the acrophase was reduced in sleep-deprived subjects.

Recent studies suggest that melatonin may have a physiological role in modulating metabolism. For example, removal of the pineal gland decreased the responsiveness of several insulin-dependent hepatic kinases during the dark phase in rats.[162] This effect was reversed by nocturnal supplementation of melatonin in the drinking water. Chronic melatonin supplementation in drinking water also reduced body weight in rodents.[163,164] Patients with metabolic syndrome have disturbances in melatonin production, characterized by an alteration of the night melatonin-insulin ratio of unclear clinical significance.[165] In a large genome-wide association studies conducted in individuals of European origin, certain genetic variants of the melatonin receptor 1B were associated with an increased risk of type 2 diabetes.[166–168] However, the most common receptor variant did not influence the relationship between sleep abnormalities and type 2 diabetes.[166] Interestingly, the melatonin receptor 1B is present on pancreatic β-cells, and melatonin has an inhibitory effect on insulin secretion. Thus, it was feared that treatment with melatonin in subjects with diabetes would increase blood glucose levels. On the other hand, since type 2 diabetes is associated with reduced melatonin levels toward the end of the night;[169] thus, there may be a physiological rationale for melatonin supplementation at bedtime. Few studies have addressed these questions. Sleep efficiency improved after five months of treatment with 2 mg prolonged-release melatonin administered two hrs before bedtime, while total sleep time was unaffected in 26 subjects with type 2 diabetes.[170] HbA1c levels also improved (8.47% *vs.* 9.13% at baseline). Lack of a control group prevented understanding whether

improved HbA1c levels were in part due to other factors than melatonin administration, such as better pharmacological and diet compliance due to study participation. In lean subjects, melatonin administration at night for 2months was associated with decreased LDL levels and improved oxidative status.[171] Another study found a positive correlation between plasma melatonin levels at 2 am and HDL levels in 36 women treated with 1 mg melatonin for one month.[172] As melatonin promotes sleep, the decrease in obesity and improvement in insulin resistance could be due to longer and better sleep *per se*, but it could also relate to other melatonin-dependent pathways yet to be identified (**Fig. 3**).

Discussion and future directions

Epidemiological and laboratory data prove the existence of a relationship between short or disturbed sleep and adverse metabolic outcomes. In general, these effects are small in size, but certain specific populations may be more susceptible than others. For example, age plays a clear role as the relationship between short sleep and obesity is stronger in children. Sleep duration and sleep architecture change with age in healthy individuals, with SWS becoming less common with age.[173] It is also well known that sex and reproductive hormones affects sleep regulatory mechanisms[174] and that sleep disorder affects women differently from men.[175] It is unfortunate that experimental studies, in both humans and in rodents, are mostly conducted in the male gender, thus unduly discriminating the female gender.

Similarly, sleep deprivation is more common in minorities but the effects of socioeconomic status *per se vs.* ethnicity remain to be determined. Individual susceptibility within these categories varies greatly: identification of contributing factors will greatly help prevention and treatment and be one of the tenets of individualized medicine.[176] Few negative effects are expected to be observed in individuals with voluntary sleep curtailment (natural short sleepers), in contrast to individuals with short sleep due to social or work-related pressure or chronic insomnia. Current epidemiologic studies often lack an objective measurement of sleep duration, such as actigraphy or repeated sleep diaries. Thus, prospective studies with objective sleep duration measurements are warranted in different subgroups to further explore this relationship.

In addition, the causality of the relationship between sleep and metabolic disorders remains unclear, even in prospective studies. Obese/diabetic individuals may be sleeping less than lean subjects because of differences in life style or stress; their sleep could be interrupted more often by symptoms of disturbed glucose levels or sleep apnea; evidence arises that possibly circulating hormones affect the brain inducing worsened sleep (**Fig. 3**). As already mentioned, causality can be addressed best in long-term studies where “prescribed” sleep duration is randomized, but this may present with practical difficulties

and significant challenges, including the fact that the biological need for optimal sleep duration varies and cannot be estimated at an individual level. Sleep is regulated by circadian and homeostatic mechanisms. As far as homeostatic mechanisms are concerned, it has been the matter of a long scientific debate whether during wakefulness, single or rather multiple metabolites accumulate, progressively increasing the pressure to sleep during the day. Classic experiments of parabiosis seem to support the notion of endogenous "sleep promoting" substances. Recently, the importance of adenosinergic neurotransmission in the regulation of sleep, especially non-REM sleep, has been underlined.[177] Nevertheless, it is certain that there is a large individual variability in sleep needs. Sensitive and specific biomarkers of sleep deprivation are needed for various reasons, including clinical and medico-legal reasons.[178]

The effect of sleep extension in subsets of short sleepers on metabolism can be addressed in randomized controlled trials. Currently, we are conducting such a study in short-sleeping obese individuals.[179] Investigating the effects of sleep extension in other subgroups of varying age, BMI, and ethnicity will also be of interest. The effect of metabolic disturbances on sleep, such as hyperinsulinemia or increased circulating levels of proinflammatory cytokines, may be empirically addressed in polysomnographical studies. As it is difficult to artificially mimic the internal milieu of obese/diabetic individuals, effects of metabolism may be modeled in animal studies using parabiosis, connecting circulations of lean and obese animals and comparing sleep characteristics.

As indicated by studies of sleep deprivation, the mechanisms connecting short sleep and obesity/ insulin resistance are probably mediated by three pathways: increases in appetite, decreases in EE, and influences on glucose metabolism **(Fig. 3**). Endocrine mechanisms influencing these pathways are complex and interconnected. The existing studies of sleep deprivation have generated variable results; small sample size, differences in the control of food-intake and physical activity levels, and inherent individual differences may have all contributed to this variability. In addition, these studies only addressed the effects of acute sleep deprivation and were not performed in real-life situations. We suggest that future studies should focus on the effects of sleep deprivation in specific patient populations, including obese/insulin-resistant individuals and chronic insomniacs *vs.* natural short sleepers, and should mimic real-life conditions.

Most studies find worsened glucose tolerance after sleep curtailment, which may be beneficial from a teleological perspective, at least in the short term. When our ancestors remained awake during the night, they were probably experiencing a threat (e.g., absence of food, presence of predators), so inducing a state in which glucose availability was advantageous. Likewise, increases in appetite result in more food intake. Together with reduced EE, this will further increase blood glucose availability. In modern societies, however, prolonged exposure to such a state may result in obesity and type 2 diabetes.

At this point, there is a general consensus that short sleep has negative effects on health, but the amount of sleep curtailment causing metabolic disturbances is unclear, and specific groups at greater risk are yet to be identified. Whether melatonin would benefit obese/diabetic subjects, or a subset of these individuals, remains to be proven in rigorous randomized clinical trials. Supplementation of melatonin has been shown to improve sleep efficiency, and HbA1c levels in patients with type 2 diabetes.[170] Lean subjects may also profit from melatonin administration as well, as it improved lipid profiles.[171,172] Thus, melatonin appears to be a promising supplement in battling the obesity/diabetes epidemics, but controlled studies need to confirm its positive effect on metabolism. Furthermore, the long-term effects and side effects of chronic melatonin supplementation need to be examined in different subgroups.

Overall, improving sleep duration and quality is a potential tool to counteract the epidemics of obesity and diabetes. However, before sleep extension advice is translated to the clinic, more research is needed.

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Conflicts of interest

The authors declare no conflicts of interest.

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CHAPTER 3

The hypothalamic-pituitary-adrenal axis, obesity, and chronic stress exposure: sleep and the HPA axis in obesity

Eliane A. Lucassen[1,2] and Giovanni Cizza[3]

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1. Clinical Center, National Institutes of Health, Bethesda, United States.
2. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
3. Section on Neuroendocrinology of Obesity, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.

Abstract

Obesity, exposure to stress and inadequate sleep are prevalent phenomena in modern society. In this review we focus on their relationships and critically evaluate causality. In obese individuals, one of the main stress systems, the hypothalamic-pituitary-adrenal axis, is altered, and concentrations of cortisol are elevated in adipose tissue due to elevated local activity of 11β-hydroxysteroid dehydrogenase (HSD) type 1. Short sleep and decreased sleep quality are also associated with obesity. In addition, experimental sleep curtailment induces HPA-axis alterations which, in turn, may negatively affect sleep. These findings implicate that obesity, stress and sleep loss are all related in a vicious circle. Finally, we discuss new strategies to combat obesity through modulating cortisol levels in adipose tissue by 11β-HSD1 inhibitors or by improving sleep duration.

Introduction

The National Health and Nutrition Examination Survey reported that in 2009–2010, 33% of adult Americans were overweight (body mass index (BMI) of 25.0–29.9 kg/m^2) and 36% were obese (BMI ≥30.0 kg/m^2) – a vast increase from 1960 to 1962 – when a similar percentage of Americans were overweight (31%), but only 13% were obese.[1] Obesity leads to significant health problems impacting nearly every organ system, including the cardiovascular system and the endocrine pancreas, causing type 2 diabetes. In parallel to the obesity epidemic, society has become increasingly fast-paced, presenting us with numerous stressors including lack of restorative sleep. In addition, self-reported sleep duration has declined by 1.5 to 2 hrs during the last 50 yrs in America.[2] Epidemiological and experimental research accumulates that both the stress system[3] and sleep quality and duration[4] are affected in obese individuals.

According to a recent meta-analysis including a total of 23,372 individuals, psychological stress is positively correlated with measures of adiposity one to 38 yrs later.[5] The overall effect size was relatively small (r=0.014; 0.002–0.025) and effect sizes were larger in men than women (0.024 *vs.* 0.017), after longer follow up, and in studies of higher quality. The relationship between habitual short sleep (generally less than 5 hrs per night) and obesity was further demonstrated in a meta-analysis of 45 cross-sectional studies, including 604,509 adults (OR 1.55; 1.43–1.68).[6] In addition, sleep curtailment and chronic stress are independently associated. In a sample of 1,300 subjects, perceived chronic stress was negatively associated with self-reported sleep duration.[7] It should be noted however that these observational studies cannot distinguish cause and consequence.

In this review, we will examine respectively the relationship between obesity and the HPA axis, obesity and sleep, and the HPA axis and sleep. For each of the reported associations, we will critically evaluate experimental studies in an attempt to determine causality and its direction. Finally we will assess the potential of strategies that aim to improve disturbances of the HPA-axis and to increase sleep duration for combating obesity.

The HPA-axis and obesity

Physiology and assessment of the HPA-axis

Exposure to stress elicits a highly orchestrated series of physical reactions, mainly operated by the HPA-axis, the sympathetic nervous system, and the sympathoadrenal system (**Fig. 1**).[8] The body adapts to repeated and chronic stress via homeostatic mechanisms, adjusting physiological responses and behavior in order to maintain stability.[9] The chronic wear and tear of this accommodation over the lifetime of an organism is referred to as the allostatic load.[8] The parvocellular cells in the paraventricular nucleus of the hypothalamus secrete corticotrophin-releasing hormone (CRH) in response to stressors, including physiological stressors such as fatigue or hunger, as well as stressors of psychological nature. CRH, in

turn, stimulates the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH), which induces the synthesis and secretion of glucocorticoids, mineralocorticoids and androgenic steroids by the adrenal cortex. Cortisol, the main glucocorticoid in humans, suppresses the immune system and induces glycogenolysis, as well as insulin resistance.[10] CRH and ACTH synthesis and release are inhibited by peripheral negative feedbacks mediated by cortisol (**Fig. 1**). In addition, CRH activates the sympathetic locus coeruleus-norepinephrine system and cortisol enhances neuronal excitability to norepinephrine. In turn, norepinephrine activates hypothalamic CRH in reverberating circuits.[9]

Cortisol depicts circadian rhythmicity, with a peak in the morning, followed by a gradual fall during the day, which is briefly interrupted by meals, and a nadir around 3 am.[4] Only 5–10% of cortisol circulates in active free form; cortisol binding globulin (CBG) binds most cortisol (80–90%) with high affinity and 10–15% is bound to albumin and sex-hormone binding globulin with lower affinity (**Fig. 1**).[11,12] CBG is fully saturated at cortisol levels over 500 nmol/l and also depicts diurnal variability, with a peak in the early afternoon.[12,13]

Cortisol levels can be determined in plasma, saliva and urine.[13] In plasma, usually the total cortisol levels are measured and the unbound, protein-free fraction is indirectly estimated using the Coolens' equation.[12] Measurements in saliva assess the biologically active, protein-free cortisol; hence the effects of within-subject changes or between-subject differences in CBG are eliminated. Saliva specimens are easier to collect than plasma and, because of the non-invasive collection, they are stress-free. Although urinary free cortisol refers to unconjugated cortisol, its level is proportional to the free (nonprotein-bound) cortisol in blood. Obtaining urine for free, unconjugated cortisol measurement also is stressfree, but the test requires 24 hrs. collection in the presence of acidic preservative. Salivary cortisol levels increase within 5 min after plasma increases in cortisol and although levels are 30–50% lower, under 500 nmol/l they are generally well correlated with plasma levels.[13]

Two NAPD(H)-dependent isoenzymes 11β-hydroxysteroid dehydrogenase (11β-HSD1 and 11β-HSD2) intracellularly convert the inactive cortisone to hormonally active cortisol and *vice versa* (**Fig. 1**).[14] 11β-HSD1 is expressed mainly in the liver and adipose tissue, while 11β-HSD2 exerts its function in the kidney and colon, making cortisol action tissue-specific. Cortisol and cortisone are metabolized to inactive tetrahydrometabolites (THM) by hepatic 5α- and 5β-reductase. Cortisol is converted to cortol and tetrahydrocortisol (THF, 5α-THF and 5β-THF), while cortisone is metabolized to cortolone and tetrahydrocortisone (THE) - metabolites often measured in urine. Alterations in 11β-HSD1 activity are reflected by the urinary (THF, 5α-THF+cortol)/(THE+cortolone) ratio, e.g., decreased 11β-HSD1 activity would lead to elevated THE levels and lower THF levels, thus to a decreased (THF, 5α-THF)/THE ratio.

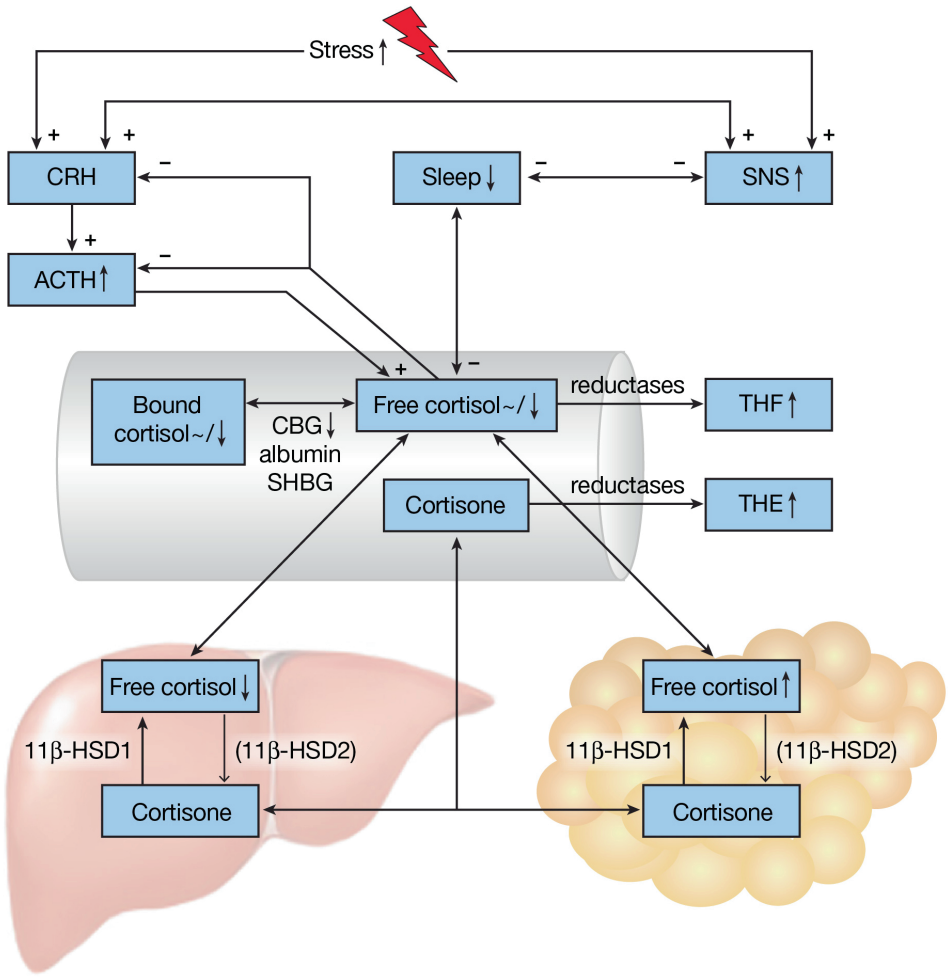


Fig. 1. Schematic representation of HPA-axis and sleep alterations in obesity. Plus and minus signs indicate positive and negative effects, respectively. Upward/downward arrows and tildes (~)depict increased/decreased and unaltered levels, respectively, of depicted phenomena in obese individuals. Obese subjects often experience an increased stress exposure, leading to increased activity of the sympathetic nervous system (SNS) and release of corticotrophin releasing hormone (CRH). CRH triggers release of adrenocorticotropic hormone (ACTH),which levels are elevated in obesity. ACTH stimulates cortisol release into the bloodstream. Cortisol, in turn, inhibits CRH and ACTH release. The HPA-axis and the SNS stimulate each other in a reverberating circuit and are able to affect sleep. Inadequate sleep is more common in obese subjects. Circulating cortisol levels, both in free form or bound to corticosteroid binding globulin (CBG), albumin or sex hormone binding globulin (SHBG),are reported unaltered of decreased in obesity. In tissues, cortisol is converted to inactive cortisone and *vice versa* by 11β hydroxysteroid dehydrogenase type 2 11β-HSD2 and 11β-HSD1, respectively. 11β-HSD1 is mostly present in the liver and adipose tissue. 11β-HSD2is present in smaller quantities in these tissues, as depicted by the thinner arrow, whereas it is abundantly expressed in the kidney and colon. Hepatic cortisol levels are decreased in obese subjects, due to decreased 11β-HSD1 levels. In contrast, cortisol levels in adipose tissue are increased due to increased levels of 11β-HSD1. Cortisol and cortisone are metabolized by reductases to the inactive tetrahydrometabolites (THM) of tetrahydrocortisol(THF) and tetrahydrocortisone (THE), respectively. Urinary levels of THM and THE are usually increased in obese subjects.

Association of the HPA-axis and obesity

Morning levels of cortisol in saliva and plasma either show no correlation, or correlate negatively with various measures of adiposity (**Fig. 1**).[15] Single-time determinations of cortisol are usually inadequate to infer the functional status of the HPA axis, given its dynamic nature: the cortisol awakening response, that is, the rise in plasma cortisol occurring 20–30 min after the habitual wake up time, is more informative to that end but is highly variable among individuals. Furthermore, cortisol measurements can be confounded by daytime stressors. Twice hourly measured plasma cortisol levels were continuously lower over the course of 24 hrs in obese individuals (mean BMI 35.4 kg/m^2) in comparison with non-obese men (mean BMI 26.8 kg/m^2).[16] In other studies, 24 hrs urine free cortisol levels did not correlate with BMI or waist circumference, but cortisol's degradation products (THM) were positively correlated with these anthropometric measurements.[17-19] Urinary 5β-THE were increased in obesity, indicating that increased 5β reduction of cortisone may be the mechanism in underlying increased cortisol clearance rate in obesity.[19] Thus, in obesity there seems to be increased turn-over (production and catabolism of cortisol) without necessarily altering circulating levels or cortisol (**Fig. 1**).

Variations in tissue-specific cortisol concentrations can exist without any changes in plasma cortisol levels through varying local 11β-HSD activity. In obese subjects, 11β-HSD1 activity has been reported either as normal[18,19] or impaired,[14] as indicated by the urinary cortisol/cortisone ratio – a rather crude and non-specific reflection, since this ratio can be altered by other cortisol metabolizing enzymes. Hepatic 11β-HSD1 activity is impaired in obesity, as indicated by decreased mRNA levels[17] and deuterated cortisol tracer infusion[19] while the enzymatic activity is selectively increased by two- to threefold in the abdominal subcutaneous adipose tissue (**Fig. 1**).[18] These human findings are mirrored by observations in animal models (reviewed in Ref 20): mice overexpressing 11β- HSD1 in white adipose tissue have unaltered plasma, but elevated corticosterone levels in the adipose tissue, and develop truncal obesity, whereas mice with liver-specific upregulation of 11β-HSD1 do not exhibit increases in adipose mass. Correspondingly, mice with 11β-HSD1 gene deficiency are less prone to develop obesity.[20] CBG was lower in single measurements in individuals with higher BMI[21] and average daytime concentrations of CBG were decreased in obese *vs.* lean subjects.[12] However, in a large survey of 477 healthy subjects there was no relationship between plasma CBG levels and BMI or waist circumference.[22] Individuals with a heterozygous $(GTTT)_n$ repeat in intron 1 of the CBG gene were more sensitive to 0.25 mg dexamethasone suppression.[23] Since CBG is expressed locally in the adipose tissue, decreased local CBG levels could enhance free cortisol exposure without changes in circulating cortisol concentrations.[21]

Mean 24 hrs plasma ACTH levels are positively related to BMI, possibly reflecting increased hypothalamic drive and/or reduced negative feedback of cortisol.[16,24] Individuals

with high BMI are less sensitive to cortisol suppression by 4 mg dexamethasone,[15] but not by lower doses of dexamethasone (0.0035 mg/kg and 1 mg).[14,25] Another study found lower cortisol suppression in women, but not in men, with 0.0035 mg/kg–0.015 mg/kg or a standard 1 mg dose of dexamethasone, indicating the association between obesity and HPA suppression could be both dose- and gender-dependent.[26] On the other hand, HPA stimulation with intravenous ovine CRH[16], ACTH[25] or mental stress tests[25] increased cortisol levels more in obese *vs.* lean individuals. Thus, the HPA-axis appears hyper-responsive to stress in obese individuals, and yet, less responsive to negative feedback, possibly to prevent free cortisol levels to fall below the normal range.

In addition, the sympathetic nervous system seems to be hyperactive in subjects with central obesity, as indicated by higher nervous discharge from the muscle sympathetic activity[27]. Increased adipokine secretion by abdominal fat tissue, including leptin and free fatty acids, may elevate sympathetic activity centrally, as adipokines are often able to cross the bloodbrain- barrier. In turn, chronically elevated SNS activity could impair β-adrenergic signalling and deregulate the HPA-axis, contributing to obesity.[27]

HPA-axis alterations in obese subjects: cause and effect?

The dramatic effects of altered circulating cortisol levels on body weight are exemplified by Cushing's syndrome (hypercortisolism due to intake of glucocorticoid drugs or increased endogenous cortisol, ACTH or CRH levels), and Addison's disease (hypocortisolism due to chronic adrenal insufficiency) presenting with central obesity and weight loss, respectively. Furthermore, visceral fat deposition increased in primates exposed to chronic physical and psychological stress.[28] In the presence of insulin, cortisol promotes triglyceride accumulation, especially in visceral adipocytes thereby leading to an increase in central fat.[29] Additionally, HPA-axis hyperactivation may lead to adiposity through increases in food intake, as artificially-induced stress, exogenous ovine CRH administration and decreased response to dexamethasone suppression led to increases in appetite, especially for more palatable foods.[30] Functional MRI studies indicate that the sensitivity of the central reward system is diminished by stress, possibly upregulating the craving for "comfort foods".[31] Interestingly, chronic stress may also lead to weight loss in a subset of individuals, possibly mediated by hyperactivation of β-adrenergic lipolytic pathways or differences in dietary restraint or coping mechanisms.[3]

In order to determine the relationship between stress and body weight regulation, the HPA-axis has been examined in obese subjects before and after weight loss. Sixteen wks of very low caloric diet induced a 29 kg weight loss which was associated with a significant decrease in 11β-HSD1 mRNA expression in adipose tissue.[32] Similarly, a four-fold decrease in 11β-HSD1 mRNA expression was reported in obese women 2 years after gastric-bypass surgery after an average weight loss of 40 kg.[33] In addition, these women had

decreased urinary THM and decreased estimated activity of 5α-reducase, indicating that the obesity-associated upregulation of 5α-reductase is an adaptive response to counteract hypercortisolism.[33] Subclinical inflammation in obesity may contribute to alterations of the HPA-axis; for example, obesity is associated with elevated levels of circulating IL-6, which in turn upregulates 11β-HSD1 expression.[34] These findings suggest that the HPA-axis alternations are both cause and effect of obesity and that they are potentially reversible to a point.

Sleep in obesity

Inadequate sleep has been associated with increased BMI (**Fig. 1**).[4,6] Obese adults (BMI: 41 ± 1 kg/m^2) without sleep apnea slept 88 min less than lean subjects, as assessed by polysomnography.[35] In prospective studies, there was a distinct but modest weight gain in short sleepers (less than 5–6 hrs) who gained 0.4 kg in a 16-year study and 2 kg in a 6-year study.[4] However, individuals with short habitual sleep durations had a 31% and 35% greater chance of a clinically significant weight gain (5 kg and 15 kg), suggesting a large inter-individual variability in this association.

Increased appetite may be a mechanism by which short term sleep loss can contribute to obesity. Increase in appetite is usually paralleled by changes in ghrelin and leptin levels, a satiety-inducing hormone secreted from adipocytes and an appetite-stimulating peptide produced in the stomach, respectively. Determining circulating levels of these neuropeptides can therefore serve as an objective measurement to indirectly assess appetite. Short habitual sleep duration, was correlated with lower levels of morning leptin and higher levels of ghrelin, which would presumably lead to increases in appetite via activation of the arcuate nucleus of the hypothalamus and subsequent increases in food intake.[36] In obese individuals however, we found no association between habitual sleep duration and morning leptin levels.[37] Obese subjects are known to display leptin resistance, which may account for these findings. As a word of caution, it should also be noted that the regulation of appetite is complex and redundant involving many neuropeptides influencing appetite, including neuropeptide Y. Short-term sleep deprivation studies can also result in insulin resistance and a pro-inflammatory state with increased plasma levels of cytokines such as IL-6.[4] Insulin resistance and increased levels of IL-6 are both associated with increased risk of cardiovascular disease and type 2 diabetes.[4]

The HPA-axis and sleep

HPA-axis dynamics and sleep

A combination of altered HPA-axis function and obesity is often observed in subjects suffering from insomnia, depression or obstructive sleep apnea.[10,16,38] A typical 8 hrs night of sleep typically consists of three to five sleep cycles; every cycle includes non-rapid eye movement

(NREM) (stage 1 to 4) and REM sleep.[4] Slow-wave sleep (SWS) encompasses stage 3 and 4 and is most prevalent in the beginning of the night, at the start of the sleep period. Patients suffering from depression often have increased cortisol and ACTH levels, hyperactivation and decreased inhibition of the HPA-axis, while their sleep is often disturbed.[10] Three years after recovery from depression, cortisol levels normalized while sleep alterations persisted.[38] In subjects with insomnia, 24 hrs plasma cortisol and ACTH and urinary free cortisol levels were all increased.[10] Obstructive sleep apnea is defined as repetitive partial or total cessations of breathing during sleep, inducing frequent microawakenings, increases in stage 1 and 2 sleep and reductions in SWS and REM sleep.[10] In obese individuals, morning levels of ACTH and cortisol were positively correlated with the amount of nocturnal apnea events.[39] Nighttime plasma levels of cortisol are elevated in patients with obstructive sleep apnea *vs.* weight-matched controls, but the ACTH response to exogenous CRH was increased, suggesting CRH hyposecretion.[16] On the other hand, hypoxia can stimulate ACTH and cortisol levels and treating the sleep apnea with continuous positive airway pressure resulted in normalization of circulating cortisol.[16]

Consequences of inadequate sleep on the HPA-axis function

Sleep onset causes an inhibitory effect on cortisol level, inducing decreased cortisol levels for 1–2 hrs after sleep onset.[10] This effect was observed when subjects were exposed to a 3-hr sleep-wake cycle or slept during the day. Inhibition of cortisol levels was also observed in "free running" conditions, when individuals have no time cues and sleep is usually initiated at a later phase of the internal circadian cortisol rhythm.[10] In addition, nocturnal awakenings transiently elevate cortisol levels, whereas morning awakening elicits a substantial and more persistent rise in cortisol levels. Slow-wave sleep is correlated with the lowest cortisol levels and lowest cortisol/ACTH response to CRH administration.[10]

Salivary and plasma cortisol were elevated in the afternoon and evening following experimental partial and total sleep deprivation, while morning levels were unchanged or even decreased.[4] Mean 24 hrs plasma cortisol levels measured every 30 min were unchanged.[4] Some studies also report a decreased cortisol peak following sleep deprivation and a slower decline of cortisol levels after its acrophase.[4] In addition, sleep may interfere with the dynamics of the HPA-axis: subjects indicating poor sleep had an exaggerated response to a combined dexamethasone/CRH challenge.[40]

Acute sleep deprivation is a stressor known to activate sympathetic activity, while decreasing the parasympathetic tone, as indicated by increased epinephrine and norepinephrine plasma levels (**Fig. 1**).[4]

Consequences of HPA alterations on sleep

Patients with Cushing's syndrome often have obstructive sleep apnea, which in turn affects sleep quality.[16,41] In addition, even patients with ACTH-dependent Cushing's syndrome without sleep apnea have an 18% decrease in sleep efficiency (ratio of time asleep and time spent in bed), patients woke up twice as often (6 *vs.* 3 times) and a 12% increase in stage 1 sleep compared to normal controls.[41] This demonstrates the direct deleterious effects on sleep of abnormally elevated levels of circulating glucocorticoids. Sleep in patients with Addison's disease has been less studied but does not present with major sleep disturbances.[42,43] Upon replacement therapy with hydrocortisone however, REM sleep duration increased in these patients.[42]

Intravenous ovine CRH or ACTH administration reduced REM and SWS.[43] Cortisol infusion also reduced REM while increasing SWS, indicating that the HPA-axis effects on REM may be cortisol-mediated, but the increase in SWS in cortisol infusion may be due to negative feedback on CRH.[43] Administration of the synthetic steroid prednisolone reduced REM, but had no effect on SWS. [43]

Battling HPA-axis alterations and sleep loss: novel therapies for obesity?

As both the HPA-axis and sleep alterations appear to influence body weight regulation and composition, targeting them to combat the obesity epidemic appears to be a viable strategy. A recent randomized control weight loss trial reported that overweight or obese African American women with self-reported high levels of stress tended to lose more weight if stress management therapy was added to a 12-week lifestyle program (2.7 *vs.* 1.4 kg); consistently, morning salivary cortisol tended to decrease to a greater extent in this group.[44] This small study had limited statistical power with only 22 women per group, but it hints that decreasing overall stress levels may contribute to weight loss.

Novel pharmacological approaches to counteract obesity through manipulations of the HPAaxis have focused on 11β-HSD inhibitors, which in principle would allow tissue-specific alterations of cortisol concentrations, without affecting circulating levels. Carbenoxolone is a non-selective inhibitor of 11β-HSD1 and 11β-HSD2. Some studies using this inhibitor report lower 11β-HSD1 expression in adipose tissue while some do not.[19,20] One study reported lower cholesterol levels and reduced glucogenolysis in healthy patients, after 7 days administration of 300 mg carbenoxolone.[45] However, 11β-HSD2 inhibition can lead to cortisol-dependent mineralocorticoid excess, as cortisol can activate the mineral-ocorticoid receptor in the kidney inducing sodium retention, hypokalaemia and fluid retention.

More selective 11β-HSD1 inhibitors, in theory void of these side effects, have recently been examined in randomized controlled trails. In patients with type 2 diabetes with a BMI between 25 and 45, daily administration of 200 mg INCB13739 for 12 wks resulted in improved insulin sensitivity and induced a larger decrease in body weight (0.9 *vs.* 0.2 kg

in the placebo group).[46] Similarly, daily ingestion of 6 mg MK-0916 and 7 mg MK-0736 for 12 wks resulted in greater weight loss in patients with a BMI over 27 kg/m^2 (1.8 and 1.4 kg more *vs.* placebo, respectively).[47] In addition, MK-0736 improved lipid profile: low- and high-density lipoprotein-cholesterol were reduced by 12% and 6%, respectively. As MK-0916 is a CYP3A4 inducer, MK-0736 may be the preferred drug of use. In all three selective 11β-HSD1 inhibitors treatment groups, concentrations of plasma androgen precursors were higher *vs.* the placebo group, but still within the normal range, suggesting mild HPA-axis hyperactivity. There was no difference in reported adverse effects in treatment *vs.* placebo groups.[46,47] In 2008–2010, there were over 70 patent submissions for 11-βHSD1 inhibitors.[48] Virtually, most major and several minor pharmaceutical companies are targeting these inhibitors.[48] In addition, there is a magnitude of pre-clinical studies examining other selective 11β-HSD1 inhibitors.[20]

Extending sleep duration in chronically sleep-deprived subjects is a challenging task. A small observational study reported that short sleepers (less than 6 hrs per night; mean BMI 27 kg/m^2) who reported having extended their sleep to 7–8 hrs 6 years later increased one point in BMI, whereas individuals who reported still sleeping less than 6 hrs per night increased their BMI by 2.1 points.[49] This study was not randomized and assessed sleep duration only at two time-points and with a questionnaire, a subjective measurement. We are currently conducting a large randomized controlled trail (ClinicalTrials.gov, identifier: NCT00261898) to examine the effect of sleep extension in a cohort of short-sleeping obese individuals.[50]

Conclusions

Subjects with obesity display alterations in both the HPA-axis and sleep. Although circulating cortisol levels are often unaltered in obese individuals, the HPA-axis appears to be hyperactive, as there are tissue-specific changes in cortisol concentrations due to differential expression of 11β-HSDs. Short habitual sleep duration and experimental sleep restriction result in bodily changes that may lead to obesity, possibly through increased appetite and food intake, but insufficient sleep also disrupts the HPA-axis. Nevertheless, studies examining the dynamics of the HPA-axis in this condition are scarce, and there is no direct information of the specific correlations of, e.g. 11β-HSD activity, circulating CBG levels and sleep, to the best of our knowledge. In turn, the activation of the HPA-axis may negatively affect sleep. Although an increased prevalence of sleep apnea or increased stress/HPA-activation is reported direct evidence is lacking for the possibility that obesity leads to worsened sleep.

As sleep loss and the HPA-axis appear to be involved in the pathogenesis of obesity, approaches aimed at modulating these systems seem viable. The generation of tissue specific11β-HSD1 inhibitors is especially promising, as this may effectively target cortisolin

selected tissues without affecting circulating cortisol levels, therefore having limited unwanted side effects. The appeal of a new class of drugs based on the mechanism summarized above is reflected by the magnitude of ongoing research. Clinical studies focusing at reducing overall stress levels or improving sleep hygiene by increasing sleep duration are currently being conducted.

In conclusion, obesity, sleep, and the HPA-axis appear to be related in a deleterious cycle with many complex interactions, including the immune system and appetite-regulation. Additional research in this field is needed to understand the alterations in these systems and to eventually combat obesity.

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This trial will give useful clinical insight in the possibility of battling obesity through sleep extension. It could be a step toward implementing individualized sleep extension advice for metabolic abnormalities in the clinical practice.


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PART I

The basic functioning of the circadian pacemaker

CHAPTER 4

Role of vasoactive intestinal peptide in seasonal encoding by the suprachiasmatic nucleus clock

Eliane A. Lucassen,[1] Hester C. van Diepen,[1] Thijs Houben,[1] Stephan Michel,[1]
Christopher S. Colwell[1,2],*and Johanna H. Meijer[1]*
*C.S.C. and J.H.M. contributed equally to this work.

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1. Laboratory of Neurophysiology, Department of Molecular Cell Biology,
Leiden University Medical Center, Leiden, the Netherlands.
2. Laboratory of Circadian and Sleep Medicine, Department of Psychiatry and Biobehavioral Sciences,
University of California, Los Angeles, United States.

Abstract

The neuropeptide vasoactive intestinal peptide (VIP) is critical for the proper functioning of the neural circuit that generates circadian rhythms. Mice lacking VIP show profound deficits in the ability to generate many behavioral and physiological rhythms. To explore how the loss of VIP impacts on the intact circadian system, we carried out *in vivo* multiunit neural activity (MUA) recordings from the suprachiasmatic nucleus of freely moving VIP knockout (KO) mice. The MUA rhythms were largely unaltered in the VIP KO mice, with no significant differences being seen in the amplitude or phase of the rhythms in light–dark conditions. Robust differences between the genotypes were revealed when the mice were transferred from light–dark to constant darkness conditions. In addition, the ability of the VIP KO mice to encode changes in photoperiod was examined. Strikingly, the behavioral and physiological rhythms of VIP KO mice showed no adaptation to short or long photoperiods. The data indicate that the intact circadian system can compensate for some of the consequences of the loss of VIP, whereas this peptide is indispensable for endogenous encoding of seasonal information.

Introduction

Circadian rhythms are present in almost all organisms, and have evolved as an adaptation to the rotation of the earth around its axis. In mammals, these rhythms are generated in the suprachiasmatic nucleus (SCN) of the hypothalamus. The SCN consists of approximately 20,000 neurons that produce an endogenous circadian rhythm in multiunit neuronal activity (MUA), reaching its peak during the day and its trough at night.

In order to function adaptively, the SCN neural activity rhythms need to be synchronized to the environment, and a variety of evidence indicates that the light–dark (LD) cycle is the main environmental signal responsible. In addition to its role in the generation of daily rhythms, the SCN is critical for the body's response to seasonal changes. In particular, the neural activity patterns in the SCN change as a function of day-length.[1-4] These photoperiod-induced changes in the patterns of SCN neural activity are thought to underlie seasonal changes in mammals' physiology and behavioral activity patterns.[4-6] Thus, the SCN is critically involved in both circadian and seasonal changes in physiology and behavior. In order to generate a coherent output signal, it is important for individual neurons within the SCN to be mutually synchronized. A major candidate implicated in interneuronal SCN synchronization is vasoactive intestinal peptide (VIP). Anatomically, VIP is expressed in neurons in the ventral, core SCN region that receive light input through the retinohypothalamic tract (RHT). These VIP-expressing cells send projections to the dorsal, shell SCN, as well as leaving the SCN and innervating SCN targets such as the subparaventricular zone.[7-9] Application of VIP alters the firing rate of SCN neurons,[10] induces Per1 expression,[11] and causes phase shifts of the SCN circadian rhythm.[12-14] The loss of VIP or its receptor (VIP receptor 2, VIPR2) disrupts neural activity rhythms of the SCN measured *in vitro*,[15-16] at least partly because of loss of synchrony of the SCN cell population.[17-18] Behaviorally, all VIP-deficient and VIP2R-deficient mice exhibit disruptions in their ability to express a coherent circadian rhythm in constant darkness (DD) as well as alterations in their ability to synchronize to the LD cycle.[17-20]

Synchronization of neurons within the SCN is also important for seasonal encoding mechanisms of the SCN.[1,3,4] In short days, the SCN ensemble rhythm is compressed and shows a narrow peak, whereas in long days, it is decompressed and shows a broad peak. The changes in the waveform pattern of the electrical SCN rhythm arise from changes in phase relationships among activity patterns of single neurons. In short days, subpopulations of SCN neurons are synchronized, whereas in long days, these subpopulations show a broader distribution.[1] An important attribute of the seasonal encoding system is its 'memory' for photoperiod. Even when animals are transferred from the photoperiod into DD, the phase relationships are preserved, and animals continue to show the characteristic behavioral patterns for days–weeks. It is not yet understood how the seasonal information is stored within the SCN, and an immediate question is which transmitter system is involved in this process.

In the present study, we first carried out *in vivo* MUA recordings from the SCN of freely moving VIP knockout (KO) mice and littermate wild-type (WT) controls. We then exposed the VIP KO mice to long (LD16:8) and short (LD8:16) photoperiods and measured the behavioral and physiological responses.

Materials and methods

Animals and housing

The VIP/PHI$^{-/-}$ mouse model was originally generated in the Department of Psychiatry and Biobehavioral Sciences at the University of California in Los Angeles. Mouse genotype was confirmed with a triple-primer PCR assay (see Colwell et al., 2003 for more information). Littermate VIP$^{+/+}$ mice were used as WT control animals in this experiment. The Animal Experiments Ethical Committee of Leiden University Medical Center approved all of the experiments.

Electrode implantation

Male mice were housed under LD12:12, LD18:6 (long day) or LD6:18 (short day) cycles. At a minimum age of 12 wks, the mice were anesthetized with Hypnorm / Dormicum and implanted with tripolar stainless steel electrodes (0.125 mm; Plastics One, Roanake, VA, USA) with a stereotaxic instrument. Two electrodes (polyimideinsulated) were used for differential recordings of the MUA, and one was placed in the gray matter for reference. Aiming for the SCN under a 5° angle in the coronal plane, the following stereotaxic coordinates were used: 0.4 mm anterior of bregma, 0.48 mm lateral to the midline, and 5.44 mm ventral to the surface of the cortex.[21] At the end of the experiment, the position of the electrode was histologically confirmed by passing a small electric current through the electrode, enabling the resulting iron deposition from the tip to be stained with potassium ferrocyanide.

In vivo MUA recordings

All mice had a recovery period after surgery of at least 1 week before placement in the recording cage with a Plexiglas front, and were connected to the recording system with a counterbalanced swivel system that allowed the mice to move freely. After appropriate amplification and filtering (0.5–5 kHz) of the signal, the action potentials were detected with window discriminators and counted in 10-s epochs. MUA data were smoothed with a penalized least-squares algorithm[22] to describe the waveform of the rhythm, and to determine peak and trough time, and peak width. Behavioral activity was measured simultaneously with passive infrared sensors. Mice were initially held for 5 days under an LD12:12 cycle followed by DD. The peak width was determined per mouse in the smoothed curve at half-maximum values and averaged.

The time of the peak and trough were determined with zeitgeber time (ZT) 12 defined as lights-off. The MUA data were smoothed, and the mean period of MUA was calculated by determining peak-to-peak time in DD per mouse. The mean amplitude was calculated per cycle, per mouse, by subtraction of trough height from peak height. The mean amplitude decrease after transfer to DD was calculated by dividing the mean amplitude levels in DD by the mean amplitude levels in the LD cycle. Phase advances in MUA were calculated by comparing the time of the 50% level of the final downward slope in the LD cycle and the half-maximum level of the first downward slope in DD, and were corrected for endogenous period.

Mice were entrained to short and long photoperiods for 25 days before recordings, as shorter entrainment periods did not lead to consistent waveform changes in the MUA rhythm in WT mice.[1] Mice exposed to long and short days were recorded for at least 5 days in the LD cycle and for approximately 5 days in DD. The peak width and phase of MUA were calculated. For mice that were exposed to long photoperiods, the quality of the rhythm decreased, and not all peaks could be used for the analysis. We only used peaks that had an amplitude of at least 30% of the amplitude in the LD cycle.

Behavioral data collection and analysis

We used passive infrared sensors to register behavioral activity during the MUA recording (**Fig. 1, Fig. 2, Fig. 4, Suppl. Fig. 2**). We made activity profiles by adding behavioral activity in 10-min bins per 24 hrs, and we made MUA profiles by adding smoothed MUA data per 24 hrs (**Suppl. Fig. 2**). For all other behavioral analyses, behavioral activity was monitored by the wheel-running activity of individually housed mice, recorded in 1-min bins (Clocklab; Actimetrics, Wilmette, IL, USA). VIP KO mice and their WT littermates were recorded for 30 days in either short or long days, and the recordings were continued for at least 14 days after release into DD. The duration of the active phase of the activity rhythm (alpha) was determined manually in individual mice by estimation of the activity onsets and offsets on each day. Amplitudes of behavioral rhythms were determined in individual mice by F periodogram analysis over the first 10 days of wheel running activity in DD. The phase advance in behavioral activity after release into DD was determined by fitting a straight line through all activity onsets, and was corrected for endogenous period.

Statistical analysis

Independent samples two-sided *t*-tests were performed in SPSS, and differences were considered to be significant if $P < 0.05$. To assess the normality of the data obtained from the *in vivo* MUA recordings, Shapiro–Wilk tests were performed. All data were normally distributed, except for the phase advance after lights off in WT mice ($P = 0.01$), so we performed a Mann–Whitney test. All values are shown as mean ± standard error of the mean. For analysis of

behavioral activity patterns, calculations of differences in alpha, in total behavioral activity and in amplitude, we used independent samples two-sided *t*-tests.

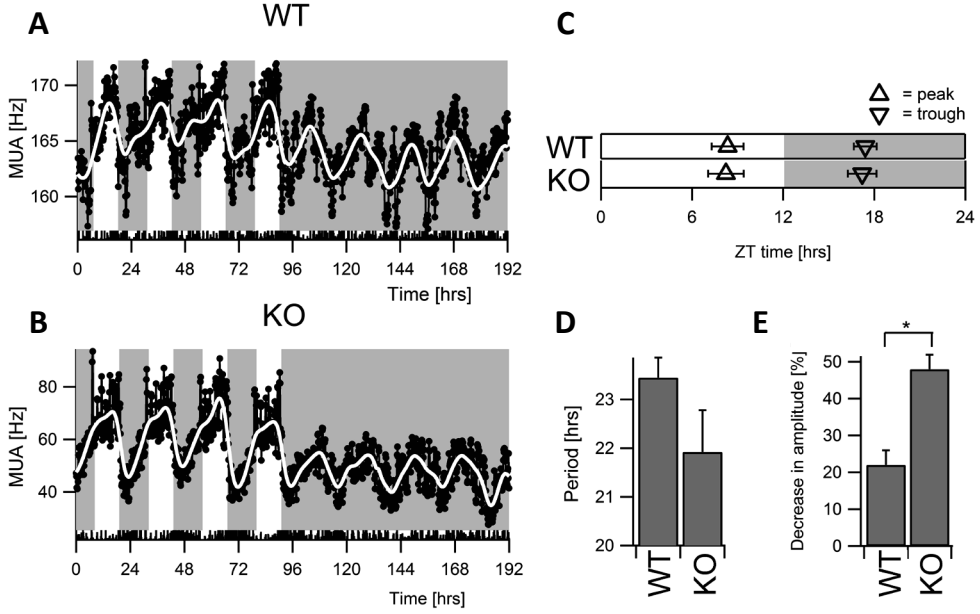


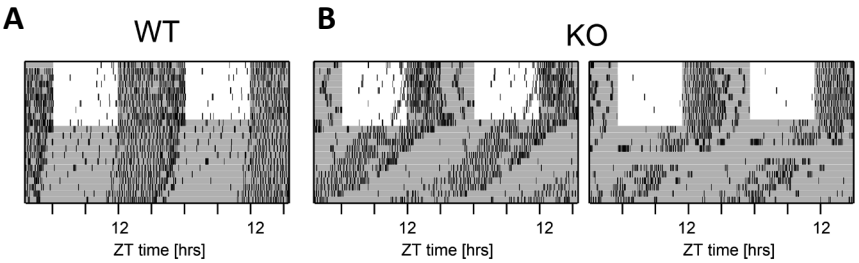
Fig. 1. SCN MUA rhythms recorded from the SCN of freely moving mice. Representative examples of MUA rhythms recorded from the SCN of a WT mouse (**A**) and a VIP KO (**B**) mouse. The mice were initially held in an LD12:12 cycle, and subsequently placed into DD. Individual data points represent 10-min epochs. Smoothed data are indicated by a white line. For this and subsequent figures, a white background indicates light exposure, and a gray background represents darkness. Behavioral activity was measured with a passive infrared sensor, and is depicted at the bottom of each plot. (**C**) The mean peak (upward triangles) and trough (downward triangles) in the MUA rhythms recorded when mice were held in LD12:12. Symbols show means ± standard errors of the mean in this and subsequent figures. (**D**) Mean period of MUA in DD in WT and VIP KO mice. (**E**) The mean (%) decrease in amplitude of the MUA level when mice were switched from LD12:12 to DD as a percentage of baseline MUA levels in each genotype. The asterisk indicates a significant difference at $P < 0.01$ measured with *t*-tests.

Results

Behavioral rhythms

Wheel-running activity was measured under LD12:12 and DD conditions to confirm the VIP-deficient phenotype observed in previous studies (see **Suppl. Fig. 1** for examples). All of the VIP KO (n = 8) and WT littermate (n = 7) mice showed clear rhythms of locomotor activity in LD conditions. When transferred to DD, VIP KO mice, in contrast to WT mice, began behavioral activity 5.59 ± 0.78 hrs earlier than what would be expected on the basis of their previous activity rhythm under LD conditions. All VIP KO mice remained rhythmic during the first 10 days in DD, as measured by F periodogram analysis. Their free-running period was

shorter (23.31 ± 0.38 hrs) than that of WT mice (23.73 ± 0.06 hrs), but this difference was not significant (*P* = 0.33).



Suppl. Fig. 1. Behavior of WT and VIP KO mice in LD12:12 and in DD. Behavior WT and VIP-deficient mice in LD12:12 and in DD. Typical examples of wheel running activity of a WT (**A**) and two VIP KO mice (**B**) in LD12:12 and consecutive release in continuous dark. Each horizontal row shows behavior over a 24 hr cycle with a white background representing light. Successive days are plotted from top to bottom.

MUA rhythms

Long-term MUA rhythms were recorded from the SCN of VIP KO (*n* = 5) and WT littermate (*n* = 6) mice (**Fig. 1A and B**). Recordings were performed for five cycles in LD12:12, and then for five cycles in DD. All of the VIP KO and WT mice showed diurnal rhythms in MUA when exposed to the LD cycle, with a peak in MUA in the middle of the day, and a trough in the middle of the night. The mean times to peak after lights-on (ZT 0) were 8.34 ± 1.05 hrs in WT mice and 8.22 ± 1.17 hrs in VIP KO mice (*P* = 0.94, *t*-test). MUA reached its trough at ZT 17.42 ± 0.76 hrs and ZT 17.21 ± 0.94 hrs in WT and VIP KO mice, respectively (*P* = 0.79, *t*-test). Under these conditions, the durations of the width of the peak in MUA measured at half maximum levels were 11.81 ± 0.32 hrs in WT mice and 12.17 ± 0.41 hrs in VIP KO mice (*P* = 0.50, *t*-test). Thus, in LD conditions, VIP KO mice were able to maintain a robust rhythm in electrical activity with characteristics and phasing comparable to WT rhythms.

All of the VIP KO (*n* = 5) and WT (*n* = 6) mice showed circadian rhythms in MUA when the mice were held in DD. In DD, VIP KO mice tended to have a shorter free-running period of the MUA rhythm (VIP KO, 21.92 ± 0.86 hrs; WT, 23.45 ± 0.41 hrs), but this difference was not significant (*P* = 0.16, *t*-test). The mean free-running periods of MUA rhythms were similar to the periods in wheel-running activity in VIP KO mice (*P* = 0.11) and in WT mice (*P* = 0.55). The width of the peak in MUA was almost identical between WT mice (11.14 ± 0.34 hrs) and VIP KO mice (11.31 ± 0.41 hrs; *P* = 0.76). There were two robust differences between the genotypes when they were transferred from LD conditions to DD. First, the amplitude of the MUA rhythm decreased more in VIP KO mice (48% ± 8%) than in WT mice (22 ± 4%, *P* < 0.01, *t*-test). Second, the MUA activity rhythm was dramatically phase advanced (**Fig. 2B**). The half-maximum level of the downward slope in MUA, which is strongly correlated with activity onset, started 5.68 ± 1.42 hrs before project lights-off in VIP KO mice and at 0.19 ± 0.58 hrs in

WT mice ($P < 0.01$, Mann–Whitney test). The start of behavioral activity of the same VIP KO mice was earlier than expected on the basis of endogenous period when lights were switched off (**Fig. 2A**). Average waveforms in MUA under LD conditions did not differ between the groups (**Fig. 2C**). We observed that the downward slope of MUA was strongly correlated with activity onset in both strains of mice (**Fig. 2D**). We made activity profiles of total behavioral activity per 24 hrs and 24 hrs. profiles of concurrently measured MUA of both genotypes (**Suppl. Fig. 2**). We calculated the level of MUA at which half of the total behavioral activity had occurred. On average, this occurred at the same level of MUA in WT mice (1 ± 0%) and in VIP KO mice (7 ± 4%, $P = 0.28$, *t*-test).

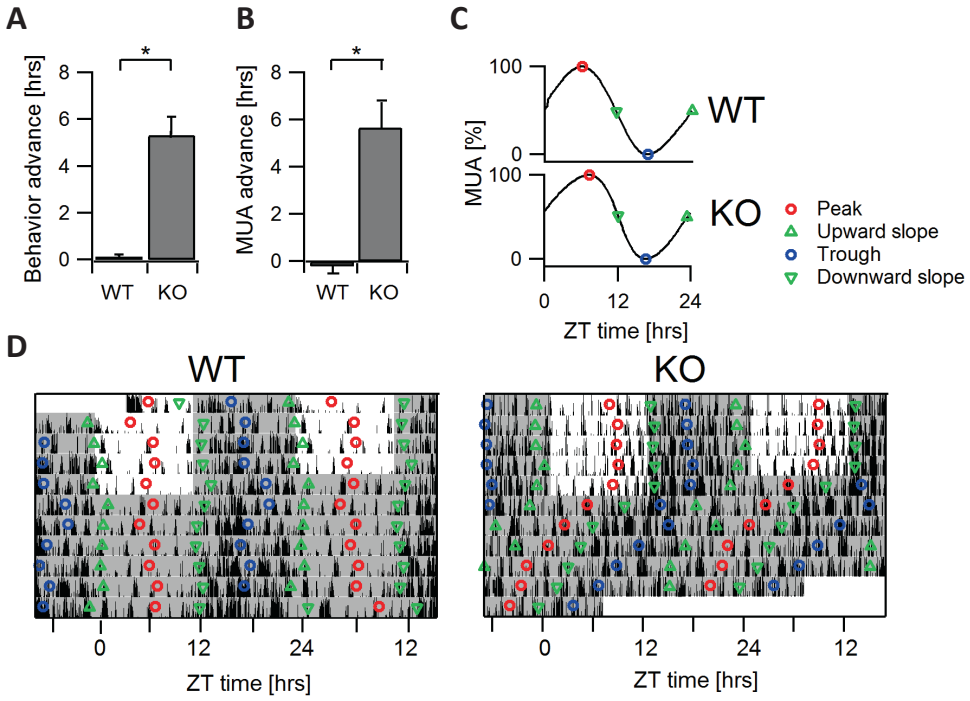
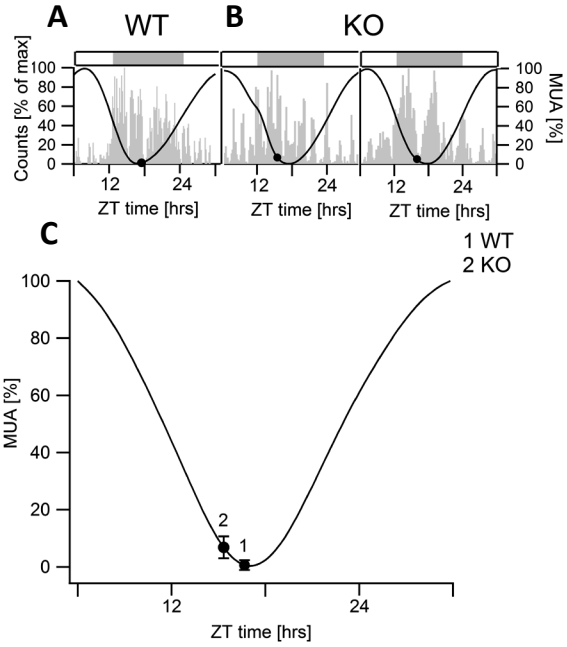


Fig. 2. Phase advance in behavior and MUA after release in DD. Bar graphs compare the phase advance in behavior (**A**) and MUA (**B**) when a mouse was released into DD from the LD cycle. The asterisk indicates a significant difference at $P < 0.01$ measured with *t*-tests (**A**) or Mann–Whitney tests (**B**). (**C**) The average waveforms of MUA in WT and VIP KO mice in LD12:12, made by adding smoothed MUA activity waveforms of all mice used for analysis. Peaks and troughs in MUA are displayed as red and blue circles, respectively. Half-maximum values of MUA are indicated by green triangles. (**D**) Representative example of behavioral activity of WT (left panel) and VIP KO (right panel) mice measured with a passive infrared sensor. Each horizontal row represents a double-plotted 24 hrs. day, with successive days plotted from top to bottom. Reference points (see legend) from the recorded MUA rhythm are superimposed on the locomotor activity plot.



Suppl. Fig. 2. Correlation between behavior and MUA in WT and VIP KO mice in LD12:12. Correlation between behavior and MUA in WT and VIP-deficient mice in LD12:12. Typical examples of activity profiles of a WT mouse (**A**) and of two VIP KO mice (**B**). Behavioral activity as measured by passive infrared sensors is depicted in grey in 10-minute bins as a percentage of maximal activity per bin size. The black curve shows normalized mean multiunit electrical activity (MUA). Light is depicted at the top of the plot with grey indicating darkness. The point in time and relative to MUA value where 50% of total behavioral activity had occurred is indicated as black dots. (**C**) Mean normalized MUA values of WT and VIPdeficient mice are indicated by the black line. The mean point in time where 50% of total behavioral activity had is depicted by black dots for WT (1) and VIP KO (2) mice and were not significantly different (*t*-test, $P = 0.28$).

Behavioral and MUA rhythms measured in long and short photoperiods

The wheel-running activity of VIP KO and WT mice was measured in short (LD8:16; KO, $n = 16$; WT, $n = 17$) and long (LD16:8; KO, $n = 11$; WT, $n = 16$) photoperiods, respectively. Mice were held in the LD cycle for 30 days, and were then released into DD (**Fig. 3; Table 1**). All of the mice showed a robust daily rhythm in LD conditions, with activity increasing sharply after lights-off. The amplitude of the daily rhythm in locomotor activity was reduced ($P < 0.01$) in VIP KO mice as compared with WT controls, with the mutant mice in the long days exhibiting the lowest amplitude (**Table 1**). Total behavioral activity was significantly lower in the VIP KO mice ($P < 0.01$) than in controls, but did not differ between long and short photoperiod exposure in either genotype. Following entrainment to long days and release into DD, VIP KO mice showed a phase advance of behavioral activity of 12.74 ± 0.61 hrs as compared with the time of expected behavioral onset based on endogenous period. After short day exposure and release into DD, VIP KO mice started activity 3.85 ± 0.28 hrs earlier than expected. The behavioral phase advance in DD was significantly ($P < 0.05$) altered by exposure to the different photoperiods (**Fig. 3**). In DD, VIP KO mice showed lower amplitude rhythms than WT controls (**Table 1**).

Table 1. Behavioral parameters of mice in short (LD8:16) and long (LD16:8) photoperiods.

	Short day		Long day	
	VIP KO	**WT**	**VIP KO**	**WT**
Alpha in LD (hrs)*,†	12.8 ± 0.3	12.9 ± 0.3	6.4 ± 0.2	7.7 ± 0.1
Alpha in DD (hrs)*,‡,§	9.3 ± 0.5	12.6 ± 0.3	11.2 ± 0.6	9.0 ± 0.3
Period in DD (hrs)	23.9 ± 0.2	23.8 ± 0.2	23.8 ± 0.4	23.9 ± 0.2
Amplitude in DD (hrs)†,‡,§	0.6 ±0.1	0.9 ± 0.0	0.4 ± 0.2	0.9 ± 0.0
Phase advance (hrs)†,‡,§	3.9 ± 0.3	0.2 ± 0.1	12.7 ± 0.6	0.0 ± 0.1

*$P < 0.05$ between short and long days in WT mice; †$P < 0.05$ between short and long days in VIP KO mice; ‡$P < 0.05$ between genotypes in short days; §$P < 0.05$ between genotypes in long days.

One of the important changes in circadian behavior observed in mice held in different photoperiods is an adaptive alteration in the duration of the night activity interval (alpha) (**Fig. 3**). WT mice showed a significantly ($P < 0.05$) longer alpha when held in short days (12.9 ± 0.3 hrs) than when held in long days (7.7 ± 0.0 hrs). Similar differences in alpha ($P < 0.05$) were observed in VIP KO mice held in short days (12.8 ± 0.3 hrs) or long days (6.4 ± 0.2 hrs). As expected, when WT animals were released into DD, alpha remained significantly ($P < 0.05$) longer after short days (12.6 ± 0.3 hrs) than after long days (9.0 ± 0.3 hrs). This history-dependent change in alpha (after-effects) was lost in the mutant mice. When VIP KO mice held in short days were released into DD, the alpha of the activity rhythm was only 9.3 ± 0.5 hrs, and those that were in long days showed an alpha of 11.4 ± 0.5 hrs.

Finally, long-term MUA rhythms were recorded from the SCN of VIP KO mice held in long days ($n = 6$) or short days ($n = 6$; **Fig. 4**). Recordings were performed for five cycles in the LD condition, followed by five cycles in DD. All of the VIP KO mice showed diurnal rhythms in MUA, whether exposed to a short or a long photoperiod. The average width of the MUA peak measured at halfmaximum levels was significantly ($P < 0.001$, *t*-test) decreased when the mice were held in short day (9.82 ± 0.38 hrs) as compared with long day (14.40 ± 0.45 hrs) conditions. In three mice, MUA was measured in more than one photoperiod consecutively (**Suppl. Table 1**). The peak width of MUA lengthened with longer photoperiod exposure. When mice were transferred to DD, all of the VIP KO mice (6 / 6) continued to show clear circadian rhythms in MUA after short day exposure. In contrast, after exposure to long photoperiods, some of the VIP KO mice (2 of 6) lost the circadian rhythmicity in MUA, and the remaining mice showed weak rhythms. Therefore, we could only reliably determine period and phase advance in MUA after short day exposure: 22.96 ± 0.13 and 1.97 ± 0.81 hrs. Importantly, after exposure to DD, the prior photoperiod had no significant impact on the MUA peak width (**Fig. 4**). The mean widths of the peak were 10.93 ± 0.83 and 12.16 ± 0.77 hrs after short and long day exposure ($P = 0.37$, *t*-test). Thus, for both the behavioral and MUA rhythms, the VIP KO mice lost the photoperiod-driven plasticity in output.

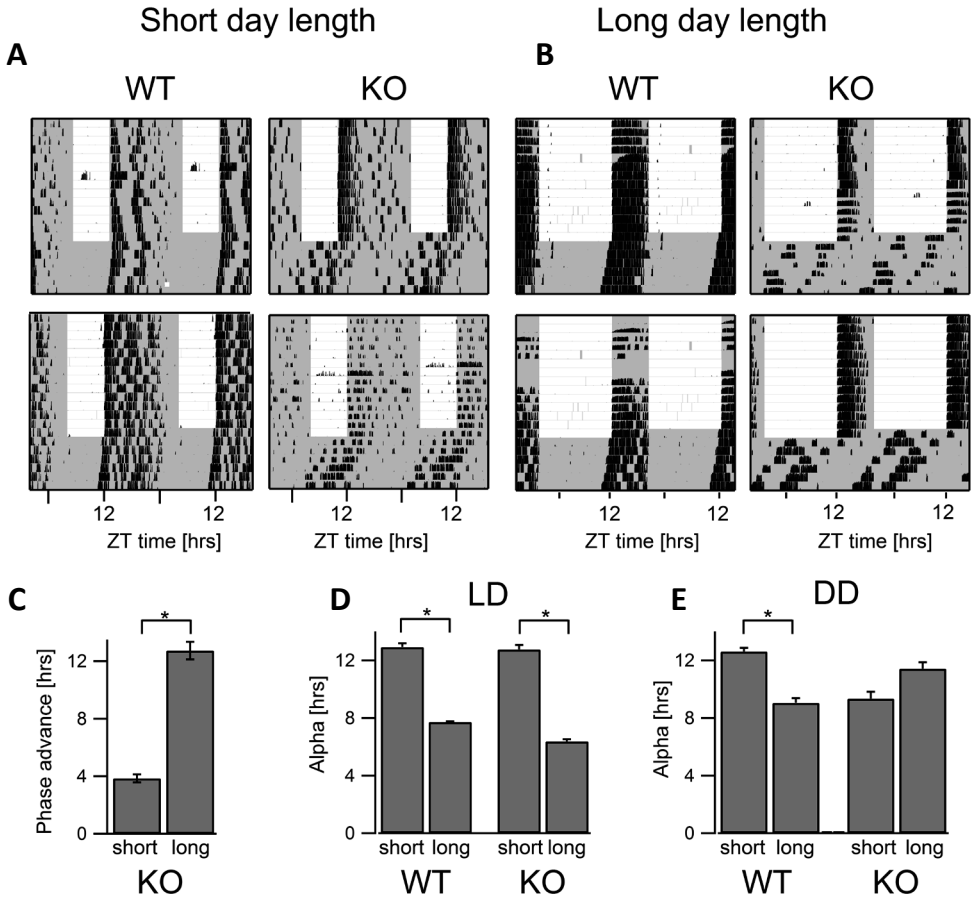


Fig. 3. Behavior of VIP KO mice exposed to short and long photoperiod conditions. Representative wheel-running activity records of WT and VIP KO mice under (**A**) short (LD6:18) and (**B**) long (LD18:6) photoperiod exposure. Each horizontal row represents wheel-running activity over a 24 hr cycle. Successive days are plotted from top to bottom. (**C**) Bar graphs plotting the behavior phase advance measured at the LD ⁄ DD transition for VIP KO mice in each photoperiod. Bar graphs showing the duration of the active phase (alpha) in each genotype under LD (**D**) and DD (**E**) conditions. The asterisk indicates a significant difference at $P < 0.05$ measured with *t*-tests.

Suppl. Table 1. Width of the peaks in MUA in a subpopulation of VIP KO mice that were measured in multiple photoperiods.

	Short day	**LD12:12**	**Long day**
Animal 1	9.04 hrs	-	13.05 hrs
Animal 2	9.54 hrs	11.66 hrs	15.51 hrs
Animal 3	-	13.76 hrs	15.51 hrs

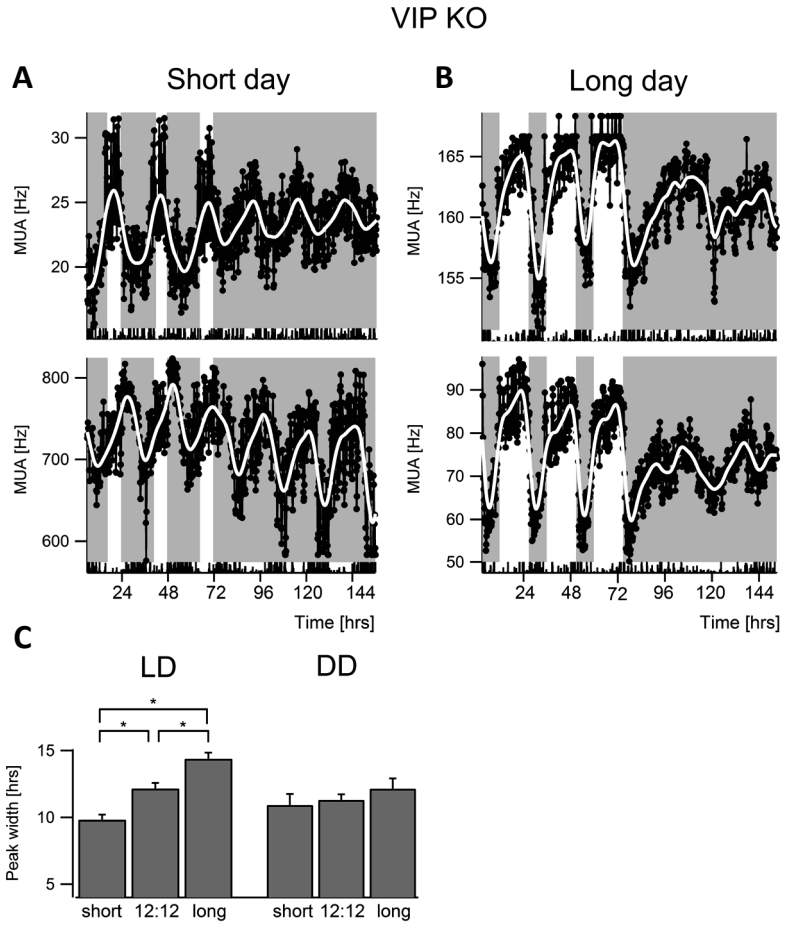


Fig. 4. MUA rhythms recorded from the SCN of VIP KO mice exposed to short and long photoperiods. Representative examples of MUA recordings from the SCN of VIP KO mice in (**A**) short (LD8:16) or (**B**) long (LD16:8) photoperiods and after release into DD. For visibility of the data, the first 3.5 days in DD are shown, but traces are part of a longer recording. Note the loss of circadian rhythmicity in the mice (B, top panel) after they had been released into DD. The white line shows the smoothed data (10-min bins). Behavioral activity as recorded with passive infrared sensors is depicted below. (**C**) The width of the peak that was measured at half-maximum electrical activity levels from each genotype. After exposure to long days, the MUA rhythms were disturbed and not all peaks could be used for the measurements. The asterisk indicates a significant difference at $P < 0.05$ measured with *t*-tests. As MUA rhythms became sloppy after long day exposure, not all peaks could be used for peak width determination.

Discussion

MUA rhythms

We implanted electrodes into the SCN of VIP KO mice and littermate WT controls, and continuously recorded MUA in freely moving mice. Under LD12:12 conditions, VIP KO mice showed strong daily rhythms in MUA that peaked during the second half of the day and reached their trough in the middle of the night (**Fig. 1**). The amplitudes and the phase of

the rhythms could not be distinguished from those recorded from the SCN of WT mice. When VIP KO mice were transferred to DD, the MUA rhythm continued, but displayed a reduction in amplitude that was significantly larger than the amplitude drop observed in WT mice (**Fig. 1**). Also, upon release into DD, the MUA rhythm of VIP KO mice showed a phase advance of about 6 hrs (**Fig. 2**), consistent with behavioral findings in earlier reports.[17,18,20] We analysed the half-maximum levels of the downward slope of MUA, as this phase of the rhythm is closely correlated with behavioral onset.[23] We found that the phase advance of the electrical activity rhythm was similar to the phase advance of the behavioral activity rhythm. We considered whether this shift reflects an unmasking of the rhythm in DD. As the electrical activity rhythm of the SCN peaked at the middle of the day under LD conditions, we have no evidence for masking of the SCN rhythm by the light. Our results indicate that, in both LD and DD conditions, the VIP KO mice show a stable phase relationship between the SCN and behavioral activity, and start their activity at the 50% level of the falling slope, as do WT mice.

The impact of VIP deficiency varies with output

As a result of recent work, it is becoming clear that the impact of the loss of VIP or VIPR2 varies with the output being measured. For example, in DD, the wheel-running activity in VIP KO mice is reduced in amplitude and coherence, and declines into arrhythmicity over the course of several weeks,[20] whereas VIPR2 KO mice are apparently arrhythmic from the start of constant conditions.[18] Rhythms in body temperature,[24-26] feeding and metabolism[27] are phase advanced but remain relatively intact. Rhythms in sleep (non-rapid eye movement and rapid eye movement) show reduced day–night amplitude and a phase advance in LD conditions.[24, 28] Rhythms in heart rate in both VIP and VIPR2 mutants are low-amplitude or arrhythmic in LD and DD conditions.[24,26] Similarly, rhythms in the blood levels of adrenocorticotropic hormone and cortisol are all lost in VIP KO mice in both LD and DD conditions.[29] With the present work, we see for the first time what is occurring at the level of the SCN *in vivo*, and find that the overall SCN neural activity rhythm is normal in LD conditions (**Fig. 1**). This suggests that we may need to look to the output pathways to explain the variability in the impact of the loss of VIP on SCN-driven behaviors. Presumably, the SCN projects to extra-SCN tissues and peripheral outputs via interneurons, particularly those that regulate autonomic outflow from the paraventricular nucleus or the brainstem, and / or those that drive the pituitary output and hence adrenal or other endocrine rhythms.[30] Many of these interneurons are VIPergic, and VIP may therefore be required for communication between the SCN and peripheral tissues to drive rhythms in behavior and other biological processes.[7,31-33]

Cellular consequences

The quality of the rhythm in MUA is remarkable, given the compelling evidence that VIP plays a critical role in coupling cells in the SCN circuit.[34] Within the SCN, genetic loss of VIP or VIPR2 disrupts circadian rhythms in neural activity in the SCN population,[15,16] where the

loss of VIP decreases the number of electrically rhythmic SCN neurons and weakens the synchrony among the rhythmic SCN neurons.[17,18] Even at the molecular level, the temporal expression of Per1, Per2 and Bmal1 is disrupted in the SCN.[35] SCN explants from VIP or VIPR2 KO mice showed low-amplitude rhythms in PER2::LUC and PER1::LUC bioluminescence that were not altered in phase as compared with controls.[35-37] Recent work by Maywood et al.[38] has demonstrated that paracrine signalling is sufficient to induce rhythmicity in VIP-deficient SCN slices, and that VIP plays a predominant role in mediating this coupling as measured by PER2::LUC bioluminescence. All of the analyses of the VIP and VIPR2 KO mice indicate that the coupling within the SCN network is greatly reduced by the loss of VIP / VIPR2 signalling, and that the VIP KO mice can be considered to have a 'broken' SCN network.

System-level compensation

The present results raise the question of what is occurring in the intact system that enables the SCN to maintain strong rhythms in MUA in the presence of reduced cellular synchrony resulting from the loss of VIP. The most obvious explanation is that light directly stimulates SCN activity when animals are kept in an LD cycle.[39,40] The decreased behavioral amplitude in DD is also explainable by an absence of entraining light signals. The melanopsin expressing retinal ganglion cells are both directly light-sensitive and receive information from rods and cones.[41-43] These melanopsin-expressing retinal ganglion cells thus encode ambient lighting[44] and generate action potentials that travel down the RHT and reach the SCN. The RHT terminals release glutamate[8] and, under certain conditions, the neuropeptide pituitary adenylate cyclaseactivating peptide.[45] The net result of RHT stimulation is an increase in the firing rate of SCN neurons. Prior work with VIP KO mice demonstrated that the direct effects of light in suppressing activity and of darkness in enhancing activity are completely unaltered by the loss of VIP.[20] Therefore, in an LD cycle, the direct effects of light and dark on MUA are likely to play a major role in maintaining the rhythmicity in SCN physiology and behavior. Previous work has also provided evidence that SCN neural activity is influenced by activity in other brain regions,[46,47] which may explain why, in VIP KO animals, the rhythms in DD are maintained *in vivo*, whereas the rhythms measured *in vitro* are severely decreased.[16,17] Prior studies with the VIPR2 KO mice have also found behavioral[48] and physiological[24] evidence that activity is a strong regulator of rhythmicity in these mice. A variety of earlier studies have noted that locomotor activity itself can modify SCN activity.[49-52] The direct regulation of SCN firing by lighting and activity levels would not be detected in the *in vitro* recording, and could be viewed as a systems-level compensation for the loss of coupling in the VIP KO mice.

Role of VIP in seasonal encoding

The SCN serves as a seasonal clock through its ability to encode daylength.[4,53,54] In line with previous literature,[1,55] the duration of behavioral activity (alpha) was broadened or

compressed when WT mice were exposed to short and long days, respectively (**Fig. 3**). Interestingly, alpha in VIP KO mice was shorter under long than under short photoperiods. When mice were transferred to DD, alpha remained highly correlated with the previous length of photoperiod in WT mice. In VIP KO mice, however, the duration of alpha was not shorter after long day exposure following release into darkness. In fact, alpha was even longer after long days, and we cannot explain this phenomenon. We therefore conclude that seasonal encoding is greatly compromised in VIP KO mice. Similar to observations in LD12:12 conditions, VIP KO mice exposed to different photoperiods showed a rapid and large phase advance with the transition to DD, but the amplitude of the phase advance was dependent on the previous photoperiods (**Fig. 3**). The phase advance was particularly large after exposure to long photoperiods, and was much smaller, and almost the same as, the behavior of WT mice following exposure to short photoperiods. The fact that the behavior of mice after short days resembled the behavior of WT mice may indicate that the short photoperiod has acted as a strong synchronizer, thereby normalizing the behavior of VIP KO mice.

To test whether the impairment in seasonal encoding in VIP KO mice is based on deficiencies within the SCN clock, we investigated the impact of photoperiod on SCN MUA waveform (**Fig. 4**). The waveform of the SCN rhythm shows major differences in response to long and short photoperiods that are brought about by changes in synchrony among molecular and electrical activity rhythms of SCN neurons.[1,3,56-59] A major question that follows from these results concerns the underlying mechanism of phase synchrony *vs.* desynchrony in seasonality. To investigate the putative role of VIP, we measured the electrical activity patterns from SCN neurons in VIP KO mice exposed to short and long days (**Fig. 4**). VIP KO mice showed a broadening of their MUA patterns when they were kept in long days, and a lengthening when kept in short days. The broadness of the peaks at half-maximum levels differed significantly between the photoperiods (14.6 *vs.* 9.8 hrs), which is similar to what is seen in WT animals.[1] Importantly, the MUA of three mice was recorded in multiple photoperiods. The results from these three recordings showed the same photoperiod-driven changes in peak width that we observed in the population (**Suppl. Table 1**). Interestingly, when VIP KO mice were transferred to DD, the width of the MUA profiles was indistinguishable between the groups from long and short photoperiods. It is well accepted that the 'after-effects' in DD indicate the storage of photoperiodic information in the SCN clock. In other words, to test the encoding capacity of the SCN for photoperiod information, the waveform of the rhythm should be evaluated after termination of the light cycle. In our results, the peak width in long days did not exceed the peak width in short days, and in fact it became narrower. The absence of any after-effect on the electrical activity rhythm therefore suggests a total absence of seasonal encoding capability of the SCN in the absence in VIP.

Although photoperiod did not affect the width of the peak, it did affect the phase advancing response of the MUA rhythm when the mice were transferred to DD (**Fig. 3E**). The

phase advance was about 10 hrs after exposure to long days and about 2.5 hrs after exposure to short days. These findings were consistent with the magnitude of the behavioral advances that we observed in VIP KO mice. Finally, we note that the overall quality of the rhythm in both behavior and in MUA activity, as indicated by the amplitude, was better after exposure to short photoperiods than after exposure to long photoperiods. Possibly, the short days functioned as a strong synchronizer, resulting in an absence of a strong phase advancing response and a diminished attenuation of the rhythm in darkness.

Conclusion

The robustness of the rhythm in electrical impulse frequency of the SCN *in vivo*, as compared with previous *in vitro* recordings,[16,17] indicate that communication between neuronal networks can compensate for the absence of VIP-induced synchrony among SCN neurons. The SCN MUA is acutely increased by retinal input,[40] and we speculate that the direct effect of light boosts the magnitude of the MUA rhythms. Possibly, light input maintains phase coherence via the RHT or through behavioral feedback effects, via the geniculohypothalamic input or the median raphe. Additional regulatory effects of behavioral activity and sleep from other brain regions on SCN activity are also likely, [46,49,50] and need to be further explored. Prior work has provided elegant evidence that SCN intercellular coupling provides robustness in the circadian system against genetic perturbations.[60] Here, we show that the intact SCN circuit can overcome the dysfunction caused by the loss of intercellular coupling in the SCN. To put it another way, as important as VIP is for the coupling of SCN neurons, the intact system appears to be able to compensate for its loss, at least under some lighting conditions. However, our findings also indicate that there are some functions that require VIP-mediated intercellular coupling, for example the reorganization of the SCN circuit that is part of the adaptation to changes in photoperiod. Without VIP, both the behavior and the physiology of the SCN appear to be unable to adapt to seasonal changes, and the 'memory' for photoperiod is lost. This is an important finding in the search for mechanisms underlying the consolidation of phase synchrony within the SCN.

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CHAPTER 5

Caffeine increases light responsiveness of the circadian pacemaker

Hester C. van Diepen,[1] Eliane A. Lucassen,[1] Roman Yasenkov,[1] Inske Groenen,[1] Adriaan P. IJzerman,[2] Johanna H. Meijer,[1,*] and Tom Deboer[1,*]
* Authors contributed equally

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1. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
2. Division of Medicinal Chemistry, Leiden Academic Centre for Drug Research, Leiden, the Netherlands.

Abstract

Caffeine is the most commonly used psychoactive stimulant worldwide. It reduces sleep and sleepiness by blocking access to the adenosine receptor. The level of adenosine increases during sleep deprivation, and is thought to induce sleepiness and initiate sleep. Light-induced phase shifts of the rest–activity circadian rhythms are mediated by light-responsive neurons of the suprachiasmatic nucleus (SCN) of the hypothalamus, where the circadian clock of mammals resides. Previous studies have shown that sleep deprivation reduces circadian clock phase-shifting capacity and decreases SCN neuronal activity. In addition, application of adenosine agonists and antagonists mimics and blocks, respectively, the effect of sleep deprivation on light-induced phase shifts in behavior, suggesting a role for adenosine. In the present study, we examined the role of sleep deprivation in and the effect of caffeine on light responsiveness of the SCN. We performed *in vivo* electrical activity recordings of the SCN in freely moving mice, and showed that the sustained response to light of SCN neuronal activity was attenuated after 6 hrs of sleep deprivation prior to light exposure. Subsequent intraperitoneal application of caffeine was able to restore the response to light. Finally, we performed behavioral recordings in constant conditions, and found enhanced period lengthening during chronic treatment with caffeine in drinking water in constant light conditions. The data suggest that increased homeostatic sleep pressure changes circadian pacemaker functioning by reducing SCN neuronal responsiveness to light. The electrophysiological and behavioral data together provide evidence that caffeine enhances clock sensitivity to light.

Introduction

Caffeine is the psychoactive stimulant that is most commonly used to reduce sleepiness worldwide. It mainly acts as an adenosine receptor antagonist, and disrupts sleep and increases alertness in many mammals.[1] Adenosine is one of the substances thought to be involved in the homeostatic regulation of sleep.[2] Its levels in the brain increase in the course of sleep deprivation and decrease during recovery sleep.[3] Recent evidence indicates that vigilance state changes affect neuronal activity in the suprachiasmatic nucleus (SCN), the location of the mammalian circadian clock. SCN neuronal activity is dependent on the arousal state of the animal, and decreases in response to an increase in homeostatic sleep drive induced by sleep deprivation.[4-6] These results suggest that circadian clock functioning may be modified by an increase in homeostatic sleep drive and thus by changes in sleep pressure. Light is the most important environmental cue for entrainment of the biological clock. Photic information reaches the SCN via the eyes through the retinohypothalamic tract. Retinal ganglion cells project via this monosynaptic pathway to the SCN. Following activation by light, glutamate is released at the nerve terminals,[7,8] leading to an increase in SCN neuronal activity.[9-16] Application of glutamate mimics the effect of light on the SCN.[8] Light exposure at the beginning of the night delays the circadian rest–activity rhythm, and sleep deprivation attenuates this phaseshifting effect.[17,18] Interestingly, administration of a selective A1 adenosine receptor subtype agonist reduces the size of the phase shift induced by light similarly to sleep deprivation.[19-21] This effect is blocked by administration of an A1 adenosine receptor antagonist.[20,21] Administration of an A1 adenosine receptor agonist decreased light-induced expression of c-Fos. These findings suggest a role for adenosine in modulating the effect of sleep deprivation on the phase-shifting capacity of the circadian clock in response to light. The current study focused on the effects of disturbed sleep and caffeine on the light responsiveness of the circadian system. We recorded SCN electrical activity in freely moving mice, and examined the effect of sleep deprivation on the response of SCN neuronal activity to light pulses. To modulate this activity pharmacologically, we studied the effect of the non-selective adenosine receptor antagonist caffeine on the light-induced changes in SCN neuronal discharge after sleep deprivation. Finally, we determined the effect of caffeine in the drinking water on changes in the endogenous period of the rest–activity rhythm in constant light (LL) and constant darkness (DD).

Materials and methods

Animals

All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (The Netherlands). All experiments were carried out in accordance with EU Directive 2010/63/EU on the protection of animals used for scientific

purposes. Male C57/Bl6 mice (Harlan, Horst, The Netherlands; age: 3–12 months) were used for behavioral experiments and for *in vivo* electrophysiological recordings of the SCN. Mice were housed individually, and food and water were available ad libitum. The ambient room temperature was maintained at 20 ± 2 °C.

Drug preparation

Caffeine (LUMC pharmacy) was dissolved in drinking water at a concentration of 0.8 mg/mL (0.08%). This concentration was found to have significant effects on circadian activity,[22] and is equivalent to the caffeine concentration in ordinary drip coffee. During *in vivo* electrophysiological experiments, caffeine was injected at a concentration of 15 mg/kg. This concentration was shown to significantly increase waking in the following hours,[23,24] and is equivalent to approximately three cups of coffee in humans.[1]

Behavioral activity

Mice were individually housed in cages containing either a plexiglass running wheel (diameter, 24 cm) or a passive infrared (PIR) motion detector (Hugrosens Instruments, Löffingen, Germany). PIR detectors and sensors on the running wheels were connected to a ClockLab data collection system (Actimetrics, IL, USA). The numbers of wheel rotations were measured and stored on a computer in 1-min bins. Animal care and experimental procedures were carried out under dim red light conditions.

Sleep deprivation

Sleep deprivation was performed during behavioral experiment 1 and during the electrophysiological recordings. During sleep deprivation, mice were kept awake by the researcher. Every time that the mice appeared drowsy, they were mildly disturbed by noise, movement of bedding, or the introduction of new nesting material or food into the cage.[25,26] As a consequence, the mice showed low levels of behavioral activity such as grooming and low levels of locomotor activity throughout the sleep deprivation procedure.

Behavioral experiment 1

Mice (n = 8) were housed with running wheels in a light–dark (LD) cycle of 12 hrs of light and 12 hrs of darkness. After at least 10 days in LD, mice were placed in DD. On the second day in DD, the mice were either exposed to a 15-min light pulse at circadian time (CT) 14, were sleep-deprived between CT8 and CT14, or were sleepdeprived between CT8 and CT14 and exposed to a 15-min light pulse at CT14. The three conditions were applied in a randomized cross-over design. CT14 is 2 hrs after the start of the active period and the time point at which the largest phase delay can be expected.[27] Running wheel activity was recorded throughout the entire experiment.

Behavioral experiment 2

Mice (*n* = 17) were housed with PIR detectors in an LD cycle of 12 hrs of light and 12 hrs of darkness. After at least 10 days in LD, mice were placed in either DD (*n* = 9) or LL (*n* = 8). Between the 10th day and the 20th day in these conditions, the mice received caffeine (0.08%) in their drinking water.

In vivo electrophysiology

Under deep anaesthesia (ketamine, 100 mg/kg; xylazine, 20 mg/kg; atropine, 1 mg/kg), a tripolar stainless steel electrode (PlasticsOne, USA) was implanted, aimed at the SCN of a mouse by the use of stereotaxic equipment with a digital readout (Stoelting). Two twisted polyimide-insulated electrodes were aimed at the SCN for differential recording of neurons, and a third reference electrode was placed in the cortex. The electrodes were implanted at a 5° angle with the following coordinates: 0.61 mm lateral from Bregma and 5.38 mm ventral to the dura. After at least a week of recovery, the mice were placed in a custom-designed recording chamber for measurement of SCN electrical activity. In the recording chamber, the mice were connected to a flexible cable and a counterbalanced swivel system. The electrical signal was amplified and bandwidth-filtered (0.5– 5 kHz). Window discriminators were used to convert action potentials into digital pulses, which were stored for offline analysis in 2-s or 10-s epochs (CIRCAV1.9 custom-made software). During the recording, the mouse was able to move freely. The movement of the mouse was recorded by PIR detectors. All data were stored for offline analysis.

In vivo electrophysiology experiment 1

After connection to the recording chamber, the mice (*n* = 11) were placed in DD. Sleep deprivation was performed during the *in vivo* electrophysiological recording between CT8 and CT14.5, on the third and seventh days in DD. During sleep deprivation, mice were kept awake by the researcher. Every time that the mice appeared drowsy, they were mildly disturbed by noise, movement of bedding, or the introduction of new nesting material or food into the cage. Between CT13.5 and CT14.5, the mice were exposed six times to a 5-min light pulse (light source, incandescent light; light intensity, 76 lW/cm^2) with intervals of 10 min. It is well known that light exposure induces sleep,[28-32] so we verified that the mice were all active and kept their eyes open during light exposure. In the control condition, the mice were exposed to similar light pulses between CT13.5 and CT14.5 without prior sleep deprivation. The conditions were applied in a randomized cross-over design. During light exposure in both conditions, the mice were closely observed, and were kept awake by mild disturbance when necessary to ensure that, in each condition, the mice were exposed to the same amount of light.

In vivo electrophysiology experiment 2

After connection to the recording chamber, mice (*n* = 6) were placed in DD. Sleep deprivation was performed between CT8 and CT14.5, on the third and seventh days in DD. At the end of the sleep deprivation, the mice were exposed to three blocks of three 5-min light pulses. We verified that the mice were active and kept their eyes open during and between light exposures. After the first block of light pulses, the mice received a control intraperitoneal injection of 0.9% saline. Twenty to thirty minutes after the saline injection, the mice were exposed to the second set of three 5-min light pulses with intervals of 10 min between the light pulses. After the last light pulse, the mice received an intraperitoneal injection of caffeine (15 mg/kg), and 20–30 min later the mice were exposed to the third block of three 5-min light pulses. The injection volume was 0.2 mL in both conditions. Histology After the recording, the mice were killed in a CO_2 chamber, and a small electrolytic current was passed through the two twisted electrodes to mark their positions. The brains were collected and kept in a 4% paraformaldehyde solution containing ferrocyanide to fix the brain tissue and to stain the recording site. After fixation, the brain was sectioned coronally and stained with cresyl violet. The marked position of the electrode was verified by microscopic inspection.

Statistical analysis

Statistical analyses were performed with SPSS (version 20) or GRAPHPAD PRISM. Repeated measurements ANOVA, paired *t*-tests and a linear mixed model with compound symmetry covariance structure and paired *t*-tests were performed to determine significant differences. *F*-values, *t* values and *p* values are reported for each statistical test. *P* values of < 0.05 were considered to be significant. For the electrophysiological data, the average of six (experiment 1) or three (experiment 2) light pulses was used as one datapoint per mouse to decrease the variation.

Results

Sleep deprivation and behavior

The effect of sleep deprivation on phase-shifting capacity was determined from wheel-running activity recordings (**Fig. 1A-B**). Light exposure for 15 min at CT14 induced a delay in behavioral activity of 1.7 ± 0.2 hrs. When the mice were sleep-deprived for 6 hrs between CT8 and CT14 prior to light exposure, the size of the phase delay was significantly reduced, by 0.6 ± 0.2 hrs ($P = 0.04$, $t = 2.584$, degrees of freedom (d.f.) = 7, paired *t*-test, **Fig. 1C**). Sleep deprivation alone induced a phase shift of 0.71 ± 0.2 hrs.

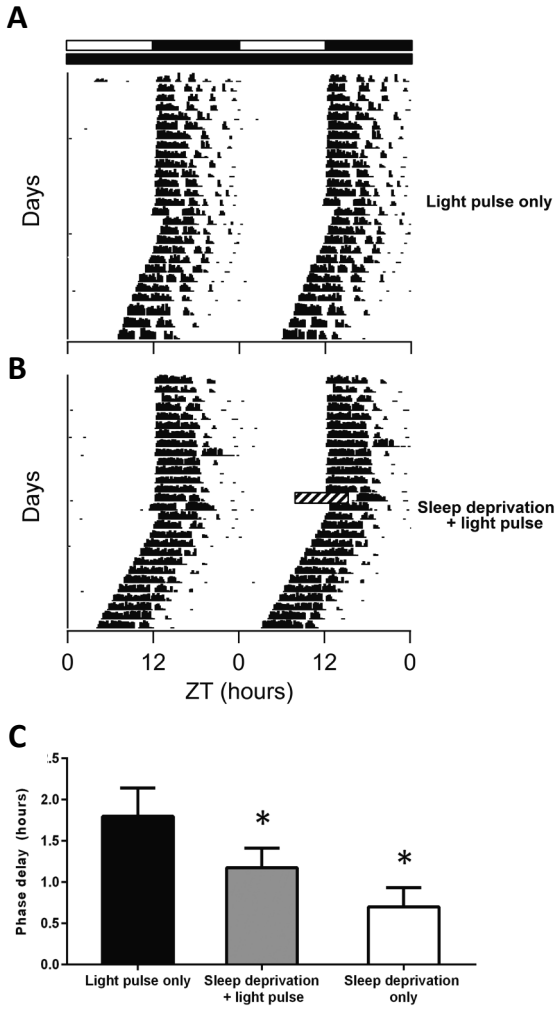


Fig. 1. (**A-B**) Two representative double-plotted actograms of mice demonstrating the phase-shifting response of running-wheel activity to a 15-min light pulse applied at CT14 on the second day in DD. Mice were either kept in DD (**A**) or sleep-deprived in DD for 6 hrs (**B**) prior to light exposure. The light regime is plotted as bars above the actograms. The striped area in B reflects the period of sleep deprivation (not double-plotted). (**C**) Mean phase shifts ± standard error of the mean of wheel-running activity of mice (n = 6) in response to a light pulse with (grey bar) and without (black bar) prior sleep deprivation; the white bar shows the magnitude of the phase shift in response to sleep deprivation alone, without light exposure. *$P < 0.05$, paired t-test. Light exposure preceded by sleep deprivation resulted in a significantly smaller phase shift. ZT, zeitgeber time.

Sleep deprivation and SCN electrophysiology

We assessed the effect of sleep deprivation on SCN neuronal activity from *in vivo* electrophysiological multi-unit activity (MUA) recordings of the SCN in freely moving mice. The location of the electrode was verified by histology (**Fig. 2A**). Successful recordings were obtained in 11 mice. In these recordings, SCN electrical discharge rates were high during the day and low during the night. All mice showed increased SCN neuronal activity in response to light (**Fig. 2B-D**). The difference between baseline MUA frequency and MUA frequency during light exposure at CT14 was 408 ± 119 Hz (**Fig. 2D**). After the mice had been sleep-deprived between CT8 and CT14, the light-induced increase in SCN electrical discharge rate was significantly reduced, to 208 ± 118 Hz (P = 0.03, t = 2.456, d.f. = 10, paired t-test) (**Fig. 2C and D**).

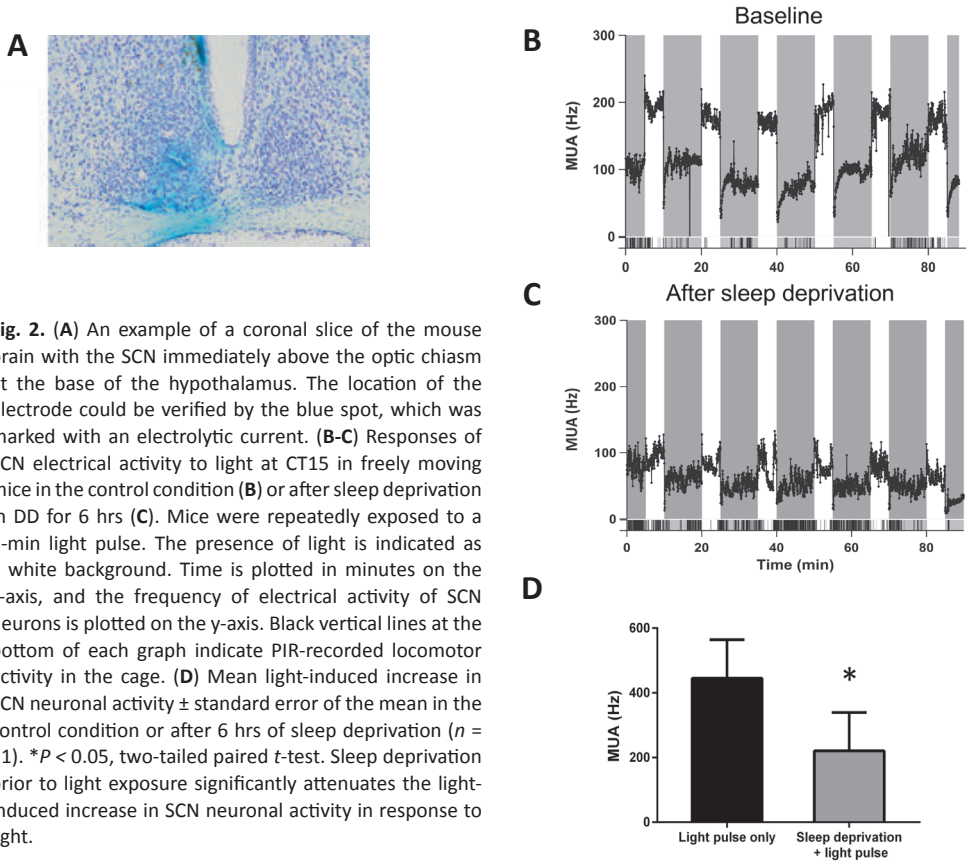


Fig. 2. (**A**) An example of a coronal slice of the mouse brain with the SCN immediately above the optic chiasm at the base of the hypothalamus. The location of the electrode could be verified by the blue spot, which was marked with an electrolytic current. (**B**-**C**) Responses of SCN electrical activity to light at CT15 in freely moving mice in the control condition (**B**) or after sleep deprivation in DD for 6 hrs (**C**). Mice were repeatedly exposed to a 5-min light pulse. The presence of light is indicated as a white background. Time is plotted in minutes on the x-axis, and the frequency of electrical activity of SCN neurons is plotted on the y-axis. Black vertical lines at the bottom of each graph indicate PIR-recorded locomotor activity in the cage. (**D**) Mean light-induced increase in SCN neuronal activity ± standard error of the mean in the control condition or after 6 hrs of sleep deprivation (n = 11). *P < 0.05, two-tailed paired t-test. Sleep deprivation prior to light exposure significantly attenuates the light-induced increase in SCN neuronal activity in response to light.

Caffeine and SCN electrophysiology

To investigate the putative role of adenosine in the reduced response to light of SCN neuronal activity after sleep deprivation, we combined sleep deprivation with administration of saline and caffeine during SCN *in vivo* electrophysiological recordings. Intraperitoneal injection of saline after sleep deprivation did not alter the response of SCN neuronal activity to light exposure (P = 0.70, t = 0.412, ratio paired t-test) (**Fig. 3A-B**). Administration of caffeine after sleep deprivation led to a significantly larger increase in SCN neuronal activity in response to light than the light-induced increase after saline injection (P = 0.004, t = 5.097, d.f. = 6, ratio paired t-test after significant repeated measurements ANOVA; P = 0.046, F = 4.66, d.f. = 5).

Caffeine and behavior

A linear mixed model was created with light, caffeine and the interaction between the two as predictors, and period length and behavioral activity as outcomes. Behavioral activity was decreased during LL (LD: 19,150 counts/24 hrs; LL: 13,398 counts/24 hrs; P < 0.001).

Caffeine did not significantly change the amount of behavioral activity (LL and caffeine: 14732 counts/24 hrs; $P = 0.07$). Overall, the mice exposed to LL had a period that was ~2.3 hrs longer than that of the mice in DD ($P < 0.001$). The effect of caffeine was highly significant; caffeine lengthened the period by ~1.6 hrs on average ($P < 0.001$). The lengthening of the period in LL by caffeine was ~1.3 hrs greater than the lengthening in DD ($P = 0.002$). Paired *t*-tests were performed to study the effects of caffeine in LL and in DD separately. In DD, caffeine treatment induced a slight increase in period, but this was not significant ($P = 0.09$, $t = -1.970$, d.f. = 7, paired *t*-test). In contrast, the period length was significantly longer in LL during caffeine treatment than in the LL control ($P = 0.01$, $t = 3.236$, paired *t*-test) (**Fig. 4**).

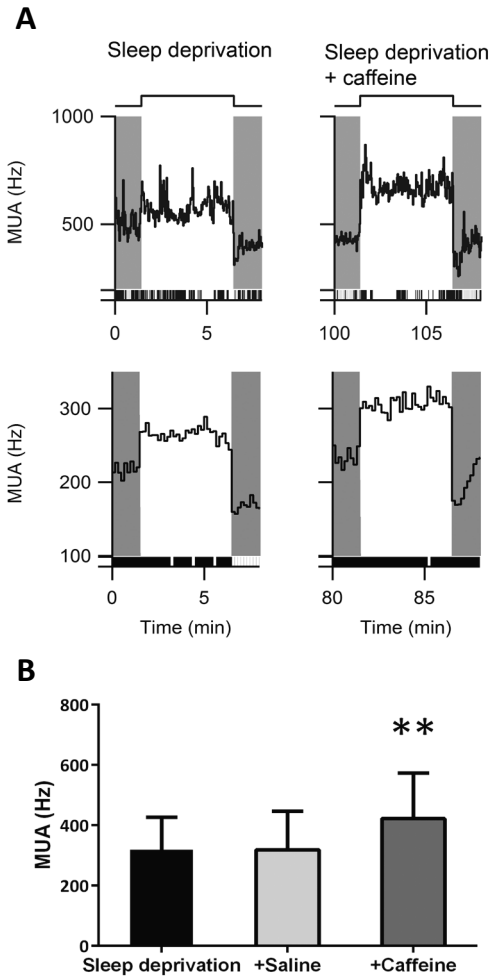


Fig. 3. (**A**) Two examples of responses to light of SCN neuronal activity after 6 hrs of sleep deprivation. Mice were exposed to 5-min light pulses. In the left graphs, light exposure was preceded by intraperitoneal injection of caffeine (15 mg/kg). Light exposure is indicated by a white background. Time is plotted in minutes on the x-axis, and the frequency of SCN electrical activity is plotted on the y-axis. Black vertical lines at the bottom of each graph indicate PIR-recorded locomotor activity in the cage. (**B**) Mean light-induced increases in SCN neuronal activity ± standard error of the mean after sleep deprivation are summarized ($n = 6$). Mice received either no injection or intraperitoneal injections of saline or caffeine (15 mg/kg), and were then exposed to 5-min light-pulses. $^{**}P < 0.01$, ratio paired *t*-tests (saline *vs.* caffeine, $P = 0.004$, $t = 0.4125$; sleep deprivation *vs.* saline, $P = 0.697$, $t = 5.097$). Caffeine significantly enhances light-induced increases in SCN neuronal activity after sleep deprivation.

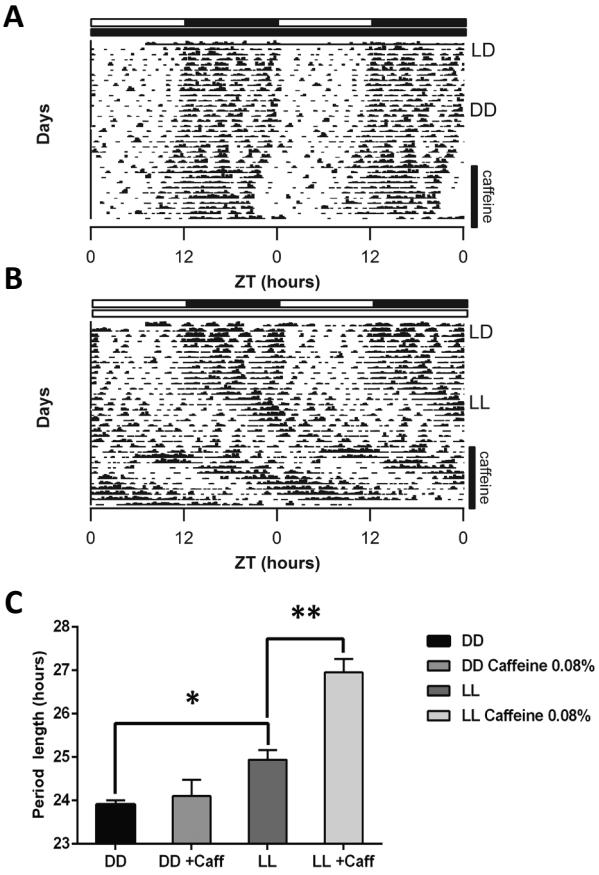


Fig. 4. (**A- B**) Double-plotted actograms of behavioral activity of mice demonstrating the effect of caffeine on free running periods in constant darkness (**A**) and constant light (**B**). The light regime is plotted as bars above the actograms. Zeitgeber time (ZT) is plotted in hrs on the x-axis, and consecutive days are plotted on the y-axis. Mice were released in either DD (**A**) or LL (**B**). After 10 days, mice received caffeine (0.08%) via their drinking water. (**C**) Mean period length ± standard error of the mean in DD (n = 8) with and without caffeine and in LL (n = 9) with and without caffeine. *P < 0.05 and **P < 0.01, mixed model with factors light and caffeine, unpaired t-test. Administration of caffeine resulted in period lengthening in both DD and LL. The period lengthening as a result of caffeine administration was significantly larger in LL.

Discussion

Our findings show that caffeine increases both light responsiveness measured at the level of neuronal activity of the SCN and the level of behavioral activity. Similarly to previous studies, we found a reduction in light-induced phase delays in behavioral activity in sleep-deprived mice.[17,18] The current study elucidates a mechanism underlying this effect at the level of the SCN. Light-induced increases in SCN neuronal activity are attenuated after sleep deprivation. We demonstrated that this attenuation was restored after peripheral injection of caffeine. We performed behavioral recordings in LL, and found enhanced sensitivity of the circadian system to light in combination with chronic caffeine treatment. Hence, the current study provides evidence for the involvement of adenosine and adenosine receptors in modulating the light sensitivity of the circadian system.

Both in the baseline condition and after sleep deprivation, the data support the notion that caffeine increases the light sensitivity of the circadian system. Aschoff's rule dictates that increasing light intensity in LL conditions lengthens the circadian period of the endogenous circadian clock in nocturnal animals.[33] Chronic caffeine consumption in DD significantly lengthened the circadian period in behavioral activity in mice, by ~25 min.[22] In the present study, we found a similar lengthening, but this did not reach significance. In addition, we showed that caffeine significantly increased the period of the circadian clock in LL, by 95 min. This is more than can be expected on the basis of the effect of caffeine alone on the endogenous period in DD. Caffeine did not significantly change the amount of activity, but there was a trend towards increased activity during caffeine consumption. Increased activity can influence the period of the rest–activity rhythm, but is normally associated with shorter periods.[34-38] The data suggest that the chronic caffeine consumption via the drinking water increased the sensitivity of the endogenous circadian clock to light, and in accordance lengthened the period of the clock.

In line with this, caffeine appeared to strengthen the effect of light on the circadian clock at the neuronal level. We confirmed that sleep deprivation attenuates phase delays in behavioral activity,[17,18] and also showed that lightinduced increases in SCN neuronal activity are attenuated after sleep deprivation. Previous behavioral experiments have indicated that the phase-shifting effects of repeated light pulses show saturation.[39] Electrophysiological experiments have shown that the response of the SCN to repeated light pulses does not diminish.[9,15,40] These results combined suggest that the saturation in the behavioral phase response occurs downstream from the initial light response in the SCN. It has been shown previously that application of an adenosine agonist attenuates the light-induced phase delays in behavior, which are restored by an adenosine receptor antagonist.[20,21] In contrast, a recent study showed that caffeine administration blocks light-induced phase delays; however, the dose applied was three times larger, and the activity of the animals was very much reduced under these conditions.[41] We found that at the level of the SCN, caffeine restored light responses. These findings show that enhanced phase shifts may have been achieved by an action of the adenosine antagonist on the SCN itself, and may provide a basis for boosting (re-)synchronization to a LD cycle.

The question remains how caffeine restores light responsiveness of the SCN. The choice of caffeine was reinforced by the preponderant use of caffeine in current society. Caffeine is an antagonist of the adenosine A1 and A2A receptors, the two major adenosine receptors in the central nervous system.[1] The density of adenosine receptors in the central nervous system is under circadian control.[42] From the present results, we cannot determine where between the retina and the SCN caffeine influences the light sensitivity of the circadian system. In the retina, adenosine receptors and ryanodine receptors (RyRs), which are caffeine- sensitive Ca^{2+} release channels,[43] are present, and can be affected by caffeine.[44-46] However, whether

adenosine levels in the retina change under the influence of sleep deprivation is unknown. Only a few studies have reported the expression of adenosine receptors in the SCN,[47,48] which turned out to be low. Despite the modest abundance of adenosine receptors, a high percentage (70%) of the SCN neurons respond to adenosine application.[47,49] This indicates that the low density of adenosine receptors in the SCN does not preclude a functional role for these receptors. In various brain regions, stimulation of the A1 adenosine receptor reduces the amplitude of excitatory postsynaptic currents and depresses glutamate release.[50-54] Previous studies have demonstrated that adenosine decreases intracellular Ca^{2+} levels and electrical activity via glutamatergic neurotransmission.[47,49] The electrical activity of SCN neurons was blocked by adenosine A1 receptor agonists, and this effect was reversed by adenosine A1 receptor antagonists. Selective adenosine A2A receptor antagonists did not affect excitatory currents.[48] Therefore, transmission of light through the optic nerve to SCN neurons is probably mediated via adenosine A1 receptors present at presynaptic nerve terminals in the SCN.[48]

The aforementioned studies provide evidence for a presynaptic influence of adenosine on glutamate release to the SCN. In our study, we blocked the effect of adenosine by the application of caffeine and restored the light-induced increase in SCN neuronal activity. A likely mechanism underlying this effect is that caffeine increases the influence of light on the SCN by blocking presynaptic A1 adenosine receptors on the retinohypothalamic tract and thereby reducing the inhibition of glutamate release to the SCN by adenosine. Another possible mechanism through which caffeine can exert its effects on the light responsiveness of SCN neurons might be via activating RyRs, which are present in the SCN.[55] Caffeine-mediated increases in Ca^{2+} levels in SCN neurons were shown to be dependent on the activation of RyRs.[56] Application of caffeine caused light-like phase shifts of the SCN in mouse brain slices, similar to the phase shifts induced by glutamate. These shifts were fully blocked by pre-incubation with RyR blockers, which indicates a role for RyRs in regulating the light-like phase-shifting effect of caffeine.[57] However, the concentrations needed to activate the RyRs *in vivo* are most likely not reached with peripheral administration of caffeine. Therefore, we hypothesize a role for adenosine receptors, in particular A1 receptors, in mediating the effects reported in our study.

Several studies in humans have reported effects of caffeine consumption on the circadian system. Melatonin release and body temperature are both used as markers of the circadian phase in humans. Caffeine ingestion suppresses melatonin release and attenuates the normal decrease in body temperature in the evening.[58] The combination of caffeine and light was shown to be even more effective in enhancing performance, reducing melatonin release, and attenuating the drop in body temperature.[58,59] suggesting that caffeine also increases light sensitivity in humans. Whether caffeine can change the endogenous period or the phase-shifting effects of light in humans remains to be determined.

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CHAPTER 6

Amplitude of the SCN clock enhanced by the behavioral rhythm

Floor van Oosterhout,[1] Eliane A. Lucassen,[1] Thijs Houben,[1] Henk Tjebbe vanderLeest,[1] Michael C. Antle,[2] and Johanna H. Meijer[1]

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1. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
2. Department of Physiology and Pharmacology, Hotchkiss Brain Institute, University of Calgary, Calgary, Canada.

Abstract

Circadian rhythms are regulated by the suprachiasmatic nucleus (SCN), a small structure at the base of the hypothalamus. While light effects on the SCN are well established, little is known of behavioral effects. This study elucidates direct modulating action of behavioral activity on the SCN by use of *in vivo* electrophysiology recordings, assessments of general locomotor behavior, and video-tracking of mice. The results show suppression of SCN neuronal activity by spontaneous behavior, the magnitude being dependent on the intensity, duration and type of behavioral activity. The suppression was moderate (32% of circadian amplitude) for low-intensity behavior and considerable (59%) for locomotor activity. Mild manipulation of the animals had reversed effects on the SCN indicating that different mechanisms are involved in the regulatory effect of spontaneous *vs.* induced activity. The results indicate that exercise at the proper time of the cycle can boost the amplitude of the rhythm of the SCN clock itself. This has potentially beneficial effects for other rhythmic functions that are under the control of the SCN.

Introduction

Mammals are equipped with a timekeeping system that enables anticipation and adaptation to environmental 24 hrs rhythms on earth. The master endogenous pacemaker is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus.[1] Individual neurons of the SCN display cyclic expression of clock genes and protein products. The protein products lead to a rhythm in membrane excitability, a rhythm in electrical activity, and rhythmic release of humoral signals, giving rise to circadian rhythms in physiology and overt behavior.[2] Synchronization of the circadian system to the environmental day and night primarily relies on external light information, transmitted to the SCN via the retinohypothalamic tract.[3]

While light is the most important cue for synchronization, the phase of the clock is also affected by the animal's behavioral activity. Arousal or increased voluntary behavioral activity, brought on by a variety of stimuli, exert changes in the circadian clock that are evidenced by period changes or phase shifts in the overt functions.[4-9] For instance, wheel running activity is known to affect the period of the clock in a dose-dependent manner[10-14] and novelty-induced wheel running elicits phase shifts of the circadian activity rhythm.[6,15] Also in humans, behavioral activity during the day accelerates phase resetting to new time zones[16-19] and physical exercise may have beneficial effects for circadian rhythm disorders related to dementia and aging.[20] A reduced rhythm amplitude is associated with aging and with a number of diseases, such as metabolic syndrome, depression, sleeping disorders and cardiovascular problems.[21,22] It is of great importance therefore to understand the potential therapeutic benefit of physical activity and study the influence of behavioral activity on the circadian system.



In vivo recordings of electrical impulse frequency in the SCN of freely moving rats and hamsters have shown that behavioral activity is accompanied by an acute suppression of neuronal activity. While potentially intriguing, these studies were limited in their behavioral analysis. It remained unknown, for instance, which type of activity can suppress the SCN, as Yamazaki and coworkers studied exclusively running wheel activity,[23] while Schaap and Meijer could not identify the type of behavior associated with SCN suppression.[24] By use of *in vivo* SCN electrical activity recordings, in combination with video-scored behavioral activity, we investigated the acute effects of various identified spontaneous behaviors on SCN electrical activity in the mouse. Additionally, we examined how the SCN responded to 'induced' rather than spontaneous behavior. We finally examined how behavioral activity at various times of the cycle affects the rhythm amplitude of the SCN clock.

Results

Suppression of the SCN firing rate

SCN electrical activity recordings were performed successfully in 14 animals and showed high levels during the subjective day (rest phase) and low levels during the subjective night (active phase). These rhythms were in anti-phase with the animal's behavioral activity rhythm in all recordings. Behaviorally-induced suppression of the SCN firing rate was observed in 9 out of 14 animals (64%). Recordings in the other animals showed no response to behavioral activity. Instead, SCN neuronal activity of 4 out of these 5 mice showed responsiveness to light, while lightinduced responses were not observed in the animals with behavioral suppressions. In the responsive mice, episodes of spontaneous behavioral activity were consistently associated with a decrease in SCN electrical activity (**Fig. 1**).

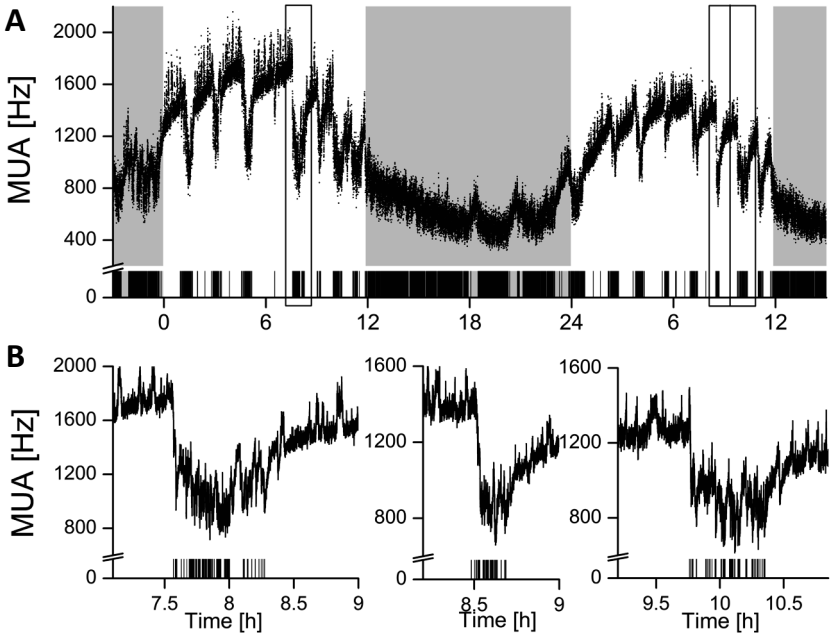


Fig. 1. SCN electrical activity is suppressed during brief episodes of spontaneous behavioral activity. (**A**) Representative example of 48 hrs recording of SCN multiunit activity (MUA) in LD12:12. Bin size is 2 sec. The grey background indicates lights off. Lower bars represent simultaneous recordings of movement (all-or-nothing modus), as obtained by a passive infrared detector. (**B**) Expanded plots of SCN electrical activity during episodes of behavioral activity at mid-day as indicated by squared blocks in A. The x-axis shows Zeitgeber Time (hrs) and the y-axis shows SCN firing rate (Hz).

Typically, at the start of behavioral activity, an abrupt drop in firing rate was observed. Firing frequencies remained suppressed for the duration of behavioral activity, and the electrical activity level gradually returned to baseline level after the animal had ceased its activity. Suppression of firing rates was observed in response to different durations of behavioral activity, even in response to episodes of activity as short as 10 sec (**Fig. 2**). An analysis of the firing patterns based on spike amplitude histograms revealed that the observed suppression is present in specific subpopulations of the SCN, while in other subpopulations it is absent (**Fig. 3**). No relation was observed with the site of the recording electrode. The suppression of SCN electrical activity occurred under LD cycles (observations from 9 animals), constant darkness (DD; observations from 7 animals) and in constant light (LL; observations from 2 animals). The magnitude of suppression reached values to up to 89% of the rhythm amplitude. The time course of recovery after an episode of behavioral activity followed an exponential pattern, and the time required to return to baseline levels was on average 15 ± 4 min (range from 5 to 21 min), irrespective of the duration of behavioral activity and circadian time.

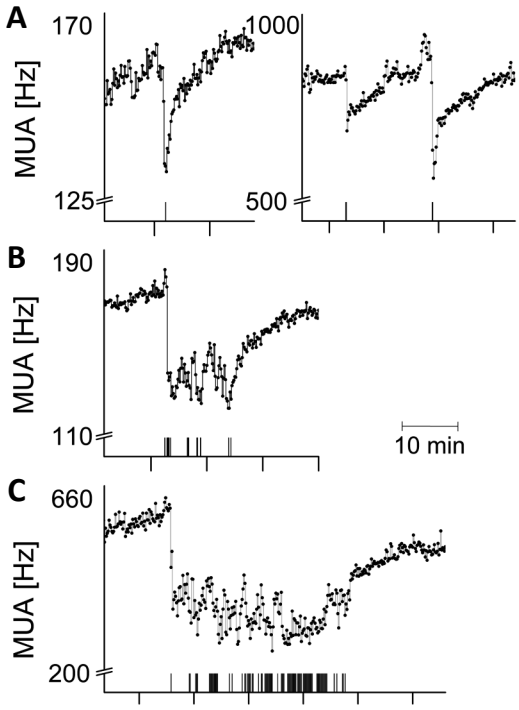


Fig. 2. Time course of suppressions of SCN electrical activity during spontaneous behavioral activity. The start of behavioral activity is characterized by an acute drop of SCN firing rate. Decreased levels of SCN electrical activity are typically sustained throughout the duration of behavioral activity. Representative examples of different durations are shown. Behavioral activity and associated suppressed levels of SCN electrical activity last for approximately (**A**) 10 sec, (**B**) 10 min, and (**C**) 30 min. Behavioral activity as detected by the passive infrared detector is plotted in the lower bar (all-or-nothing modus). Note that the PIR detector does not detect all behavioral activity which became apparent from the video recordings. Suppressions that lasted <1 min were observed in ~20% of the cases, the occurrence of suppressions of 1–25 min was ~65%, and the occurrence of long suppressions of >25 min was ~15%. In each figure, the x-axis represents time (scale unit is given by the bar in the figure). Suppressions of all shown examples occurred between Zeitgeber Time (ZT) 3 and ZT10) under LD conditions. The y-axis shows SCN firing rate (Hz). Bin size is 10 sec.

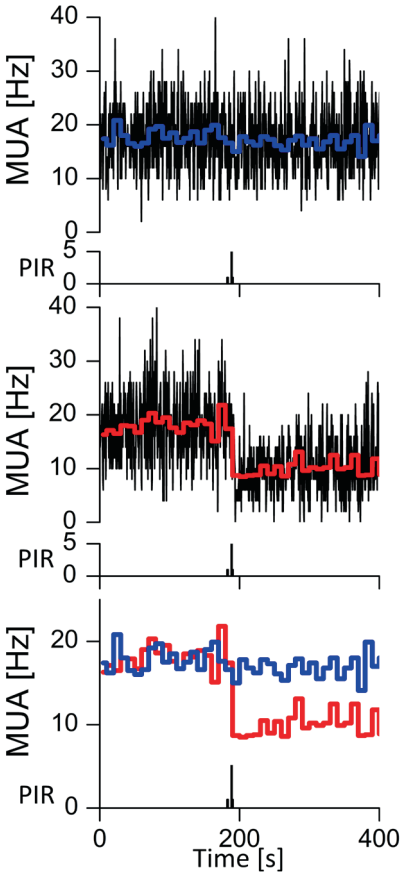


Fig. 3. Electrical activity simultaneously recorded from two SCN sub-populations. Action potential thresholds were set off-line in such a way that an average firing frequency of 18 Hz was measured during baseline, in order to obtain approximately equal-sized populations of neurons. Bin size is 0.5 sec, and lines in color represent the averaged firing frequency per 10 sec. Spikes between excluding thresholds were counted, and revealed that the decreased firing rate during behavioral activity is apparent in some neurons (**lower graph**), while it is not present in others (**upper graph**). For comparison, the averaged firing frequencies are plotted in one graph below.

Type and intensity of behavioral activity

By use of video-analyses, different types of behavioral activity could be distinguished. Four animals had long video records that included examples of all behavioral subtypes. A number of clear suppressions (n = 69) were selected from these 4 animals for analysis (n = 11 were taken from LD conditions, n = 58 were taken from DD conditions). The SCN recordings and the accompanying video recordings were analyzed (**Fig. 4**, **Suppl. Video 1**). The magnitude of the suppression, expressed as a percentage of the amplitude of the circadian rhythm, ranged from 17% to 89%. In all cases, the animal had been lying or sitting quietly prior to the suppression. The type of behavior that was associated with the acute drop in electrical activity included one of the following behaviors: grooming, moving or walking. The magnitude of suppression of neuronal activity was related to the type of the initial behavior (**Fig. 4**). 'Grooming' behaviors inhibited the SCN firing rate by 32 ± 3%, while 'moving' behaviors suppressed SCN firing by 43 ± 3%. Walking produced the strongest suppression (59 ± 3%)

of electrical activity ($P < 0.01$, ANOVA with post hoc Bonferroni test). While the behaviors 'eating' and 'rearing' were not found to initiate SCN neuronal suppression, these behaviors were often capable of maintaining electrical activity at reduced levels. Suppressed MUA levels as observed during non-intense behaviors, such as grooming, eating, drinking, and rearing, could be further decreased if they were followed by more intense behavior such as locomotor activity (**Fig. 4A**).

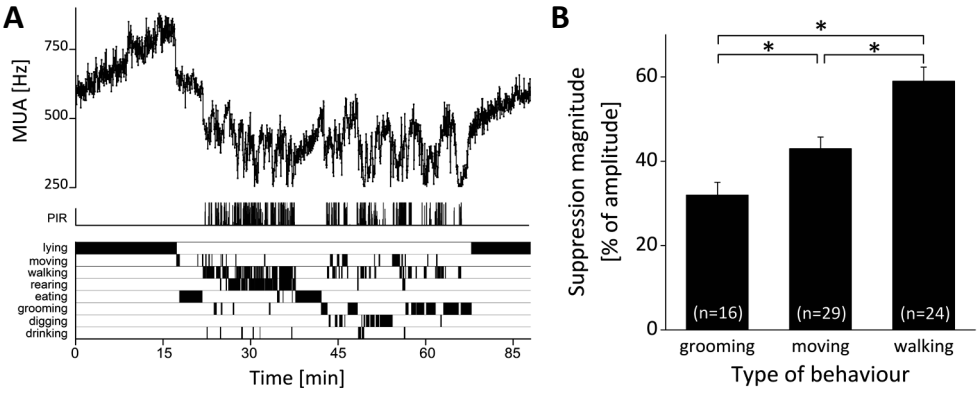


Fig. 4. Relationship between type and intensity of behavioral activity and SCN electrical activity. (**A**) The SCN electrical activity profile as recorded throughout a full episode of behavioral activity (approximately 45 min). Lower bars represent the type of behavior as scored by videoobservation. Behavioral activity as recorded by passive infrared detector is depicted at the bottom of the MUA trace in a graded scale. In this example, the initial behavior leading to a suppression of SCN activity is associated with moving, and is followed by eating which does not induce further suppression but keeps the SCN electrical activity at a reduced level. A further decrease of spike rate is induced when the animal starts locomotor behavior. While the suppression lasts for the full duration of the behavioral activity bout, gradual changes are associated with different types or intensities of behavior, and electrical activity gradually returns to baseline when the animal has ceased its behavioral activity. (**B**) The magnitude of suppression represented as a function of type of behavioral activity that initiated the suppression. Magnitude of suppression was significantly different between groups (*P = 0.01, ANOVA with post hoc Bonferroni test). N-values are given between brackets. Error bars represent the standard error of the mean.

Suppl. video 1. Suppression of SCN activity by spontaneous behavior. The animal is lying quietly before the start of the video fragment. Video recording starts at time 20 sec. The electrical impulse frequency of the SCN is depicted on the y-axis. The movements as detected by the passive infrared detector (PIR) are indicated below the electrical activity trace. At the bottom, the following behaviors were scored: lying, moving, grooming, sitting, walking and eating.
http://journals.plos.org/plosone/article/asset?unique&id=info:doi/10.1371/journal.pone.0039693.s003

Suppl. video 2. Excitation of SCN activity by induced behavior. The animal was disturbed and activated by a movement of the cage at time 60 sec. This resulted in a temporal increment of SCN electrical activity.
http://journals.plos.org/plosone/article/asset?unique&id=info:doi/10.1371/journal.pone.0039693.s004

Stimulated behavioral activity

In contrast to spontaneous behaviors, mild disturbances of the animals produced an acute transient increase of the SCN firing rate that lasted for 10 sec to 200 sec (**Fig. 5**, **Suppl. Video 2**). The increment in SCN firing rate was observed in the same animals that showed inhibitory responses to spontaneous behavioral activity (**Fig. 1**). We had 3–5 observations (induced activity experiments) per mouse. Of the total number of observations, 40% were in LD and 60% in DD, and they were measured during the subjective day (CT/ZT 12–24). During the increment in SCN firing rate, the animals often showed a freezing response, which was followed by locomotor activity. The elevated levels of SCN activity were either followed by a drop in electrical activity as compared to baseline, or they returned to baseline.

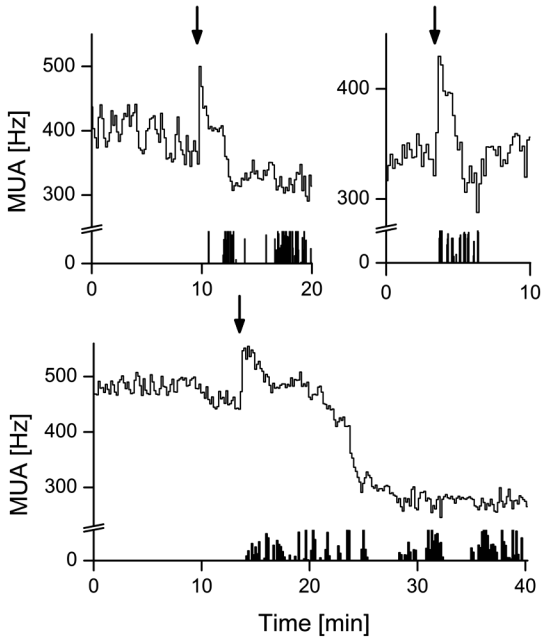


Fig. 5. SCN electrical activity in response to mild disturbance of the animal's rest during the day. The disturbance was established by making a small movement of the cage, without touching the animal. The timing of disturbance is indicated by the arrow. The increment of SCN discharge levels is acute, and returned to baseline quickly. In some cases the increment in electrical activity was followed by a suppression in electrical activity when behavioral activity continued (**lower graph**). It is possible that in these cases, induced activity had changed into motivated activity. Behavioral activity as recorded by passive infrared detector is depicted at the bottom of each MUA trace in a graded scale.

Influence on circadian amplitude

Behavioral suppression occurred at all phases of the circadian cycle (based on 229 observations from 13 cycles from 2 animals in DD and in 280 observations from 16 cycles from 4 animals in LD; mean ± SEM = 19 ± 1 observations per circadian hour). On average, the maximal occurring magnitude of neuronal suppression during the day was only slightly larger than during the night in all but one animal (averaged 47 ± 1% (day; n = 2 ± 6) *vs.* 43 ± 1% (night; n = 2 ± 3) respectively; $P < 0.05$; ANOVA). We plotted the number of action potentials as a function of the time of the cycle, and distinguished between behaviorally active and inactive episodes of the mice. We found that clear circadian rhythms exist both in

the absence and in the presence of behavioral activity (**Fig. 6**). The smoothed curves through the MUA rhythms revealed that the level of the rhythm was lowered in the presence of activity.

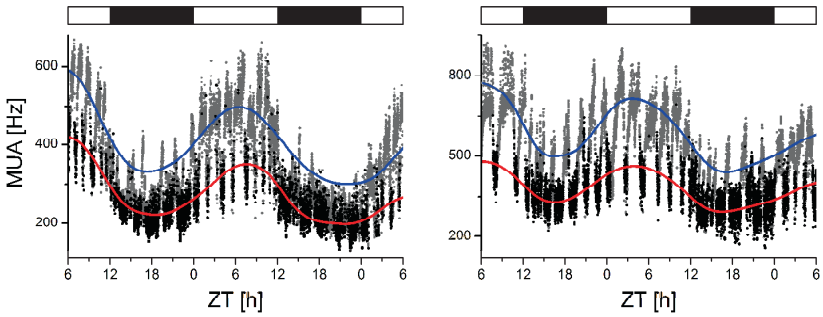


Fig. 6. Circadian profile of SCN electrical activity in the presence and absence of PIR-recorded behavioral activity. To visualize the effect of behavioral activity on SCN rhythm amplitude, the passive infrared activity data are integrated in the electrical activity data: whenever passive infrared movement was detected during a 10 sec recording bin, the number of SCN spikes counted in that bin is represented by a black dot. Grey dots show multiunit activity data points from bins when no movement was detected. Eye-fitted lines were drawn through grey dots (blue line) and black dots (red line) to illustrate the presence of a circadian rhythm in either profile. The LD12:12 light cycle is indicated above the recordings (white, lights on; black, lights off).

Discussion

By use of stationary micro-electrodes, we measured the acute effects of behavior on the SCN and show that behavioral activity itself acts as a potent stimulus that modulates SCN pacemaker activity. The SCN electrical activity was strongly affected by the animal's spontaneous behavioral activity level in an intensity- and duration-dependent manner. Electrical discharges were abruptly decreased upon the start of activity, and remained suppressed for the full duration of behavioral activity. By contrast, behavioral activity triggered by mild disturbance of the animal's rest led to excitation instead of suppression of SCN activity. The spontaneous activity of the animals during the night resulted in a decrease of the trough level at night, while inactivity during the animals' resting phase kept the peak of the SCN at high levels. Thus, a reciprocal interaction exists between overt behavioral activity and the rhythm amplitude of the central circadian pacemaker.

Identification of effective behavioral activity

Video recordings allowed for the identification of specific types of behavior that were able to induce suppression of SCN activity. We established the dependence of SCN neuronal suppression on the duration and intensity of activity. Surprisingly, ultra-short (<1 min) episodes of behavioral activity (**Fig. 2, Fig. 4**) were capable of suppressing SCN activity to

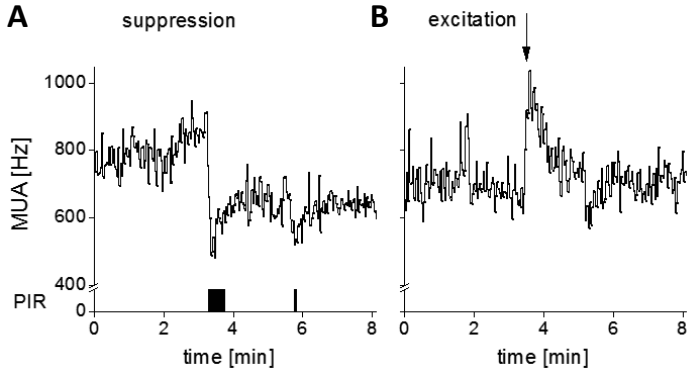
levels indistinguishable from those induced by longer activity periods. Furthermore, we found that non-intense types of behavior were capable of inducing substantial suppression (~30%) of SCN electrical discharge levels. Notwithstanding, with more vigorous types of behavioral activity, the suppression of SCN activity became larger. The strongest modulation of SCN firing rates was associated with locomotor activity (~60%; **Fig. 4**). When activity switched from one type to another, the electrical activity remained suppressed, but often the level of suppression changed, depending on whether the activity was more intense (further suppression), or less intense (**Fig. 4A**). The presence of behaviorally-induced suppression in mice is consistent with previous electrophysiological observations from rats[24,25] and hamsters.[23] In hamsters, behavioral activity leads to suppressions in all recordings[23] while in rats such suppressions were only observed in 5 out of 29 animals.[24] These differences may relate to differences in the organization of input pathways, and to differences in phase shifting effects of behavior in these species. The present results indicate that more intense types of behavior lead to larger suppressions, with locomotor activity being the most potent modulator of electrical activity.

The neuronal suppression consistently occurred during spontaneous behavioral activity in the 9 responsive animals. However, it is not clear whether the two phenomena can be causally disentangled. We believe that the suppression of activity of the SCN is inextricably bound up with behavioral activity and may be caused by it. For one reason, in the isolated SCN *in vitro*, no spontaneous suppression of the SCN neuronal activity is observed while recording techniques are comparable (i.e. stationary MUA recordings).[26] This indicates that input from, or interaction with, other brain areas is critical. Moreover, after termination of the behavioral activity, the electrical discharge recovered only slowly from suppression, while the behavioral activity was already fully arrested. Together, these findings suggest that physical activity leads to suppression of SCN electrical activity, most likely via activation of extra-SCN areas.

The direct suppressive effect of behavioral activity on SCN firing rate may relate to the finding that behavioral cues shift the circadian rest-activity cycle. The critical aspect of the behavioral stimulus causing these shifts is not fully identified. Previously, running wheel activity was considered essential to phase resetting of the clock. Indeed, a close correlation exists between hamster running wheel activity and the magnitude of a phase shift,[15] and it had been shown that phase shifts were absent or attenuated when running wheel activity was blocked.[8,9] However, sleep deprivation during the day with only minimal amounts of locomotor activity can also produce large phase shifts,[4] and less clearly defined behavioral activity patterns, such as those induced by cage cleaning, lead to phase shifts, albeit of smaller magnitude. Our results can explain these behavioral findings; while locomotor activity has the largest effect on SCN activity, other types of behaviors can also acutely alter SCN firing rates, although to a lesser extent.

Effects of induced versus spontaneous activity; response direction and kinetics

When behavioral activity was evoked by mild disturbance of the animal, the observed responses of SCN neuronal activity were transient and excitatory. These responses were opposite to those induced by spontaneous behavior observed in the same animals (**Suppl. Fig. 1**). SCN neuronal discharge levels immediately increased in response to the stimulus and remained elevated for a maximum of 200 sec (**Fig. 5**). The kinetics of the recovery of the excitatory responses was different as compared to the recovery from suppressions. Following suppression, the recovery took remarkably long (approximately 15 min) and resembled an exponential function in accordance with previous results.[24] In contrast, the recovery from the excitation was fast. These findings raise questions on the potential underlying mechanisms.



Suppl. Fig. 1. Illustration of SCN suppression and SCN excitation in the same animal. Representative example of a trace showing suppression of SCN electrical activity in response to spontaneous behavioral activity (**A**). The occurrence of suppression was set as a criterion for testing the SCN response to stimulated behavior, which appeared to be excitatory as is shown by the recording trace in (**B**). Recordings from A and B were depicted during the subjective day. Bin size is 2sec. Lower bar represents the animals spontaneous movements as recorded by a passive infrared detector. Note that the PIR sensor does not detect all behavioral activity which became apparent from the video recordings. The timing of the disturbance of the animal's rest is given by the arrow. X-axis represents time (min), y-axis represents the SCN multiunit activity (Hz).

The neuronal pathways involved in phase shifting by behavior are thought to originate from the raphe nuclei, which contains serotonin (5-HT),[27] and/or from the intergeniculate leaflet, which contains neuropeptide Y (NPY), GABA, enkephalin and neurotensin.[3] $GABA_A$ activation in the SCN mimics the effects of non-photic manipulation on both circadian phase and clock gene expression.[28] Increased behavioral activity causes elevated levels of 5-HT[29] and NPY[30] in the SCN, and leads to increased c-Fos expression in hypocretin cells of the lateral hypothalamus.[31] Hypocretin application to coronal slices induces suppression of SCN neuronal activity with characteristic slow recovery.[32] Given the time course of the onset of the suppression, which is immediate upon behavioral activity onset, any of the inhibitory

neurotransmitters could be involved, while the slow recovery at the end of the suppression would be consistent with the involvement of hypocretin or another neuromodulator.

While the procedure used to induce behavioral activity in our study could have activated a stress response, it is unlikely that a stress factor is causal to the change in SCN activity. First, the increase in SCN electrical discharge was instantaneous, whereas activation of a cortisol response would require more time. Furthermore, a similar approach for inducing behavior in hamsters has been shown to produce little cortisol response in the short term.[33] Finally, stress has little effect on the circadian system of rats and hamsters,[33,34] and corticosteroid receptors are absent in the SCN.[35,36] Instead, the excitatory SCN response may involve the participation of arousal or anxiety systems.[10,37] Neural structures that have been implicated in arousal responses include several nuclei in the diencephalon and brain stem, producing acetylcholine, histamine or norepinephrine and dopamine.[38]

Elevations in firing rate may be due to excitatory stimulation of the SCN, as well as to a transient inactivation of hyperpolarizing signals (disinhibition). Thus, the involved neurotransmitter system could be excitatory or inhibitory. The analysis of subpopulations revealed heterogeneity within the recorded population. This suggests that incoming fibers reach part of the recorded area, and indicate the presence of refined but complex input organization. Similar heterogeneity in SCN responses has also been observed in response to photic signals.[39] When disturbed, mice in our experiment often initially froze but were alert. This behavior is usually associated with type 2 theta activity in the hippocampus, which is mediated by acetylcholine.[40] Acetylcholine is known to innervate the SCN from forebrain and brainstem cells.[41] It is possible that the cholinergic regions activating type 2 theta may also regulate SCN activity.

Boosting circadian amplitude

The behaviorally-induced changes in SCN firing rate are superimposed on the SCN circadian rhythm and were found at all phases of the circadian cycle. As behavioral activity of nocturnal animals is concentrated during the night, suppression of SCN electrical activity is especially present during the night. As a result, the trough in SCN electrical activity is lowered. This finding indicates that the SCN rhythm amplitude can be boosted by behavioral activity during the animal's active phase, i.e. the phase of the cycle where SCN electrical activity is low (**Fig. 7**). Accordingly, the SCN pacemaker itself can be regarded as a node of a loop, in which SCN electrical activity and behavioral activity influence one another. When behavioral activity falls at the proper time of the cycle, i.e. when it occurs at night in nocturnal animals, the electrical activity of the SCN gains amplitude by a reduction of the trough level. When the animal is resting during the day, no suppressions will occur, keeping the peak of the SCN rhythm at a high level. Reversely, behavioral activity at uncommon phases of the circadian cycle (i.e. daytime for nocturnal animals) would result in lowered peak levels of SCN electrical activity, leading to reduced SCN amplitude (**Fig. 7**).

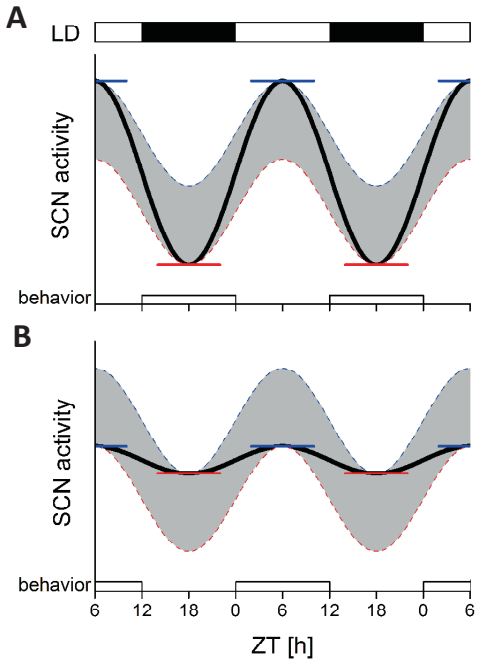


Fig. 7. Consequence of night- *vs.* day-time activity on SCN rhythm amplitude. Blue line represents the SCN activity in the absence of behavioral activity; red line represents the SCN activity in the presence of behavioral activity. Both lines are based on results shown in Fig. 6. The black lines represent two extreme possibilities: (**A**) when behavioral activity is concentrated during the night and is absent during the day, the amplitude of the SCN electrical activity is maximal. (**B**) In contrast, when behavioral activity would occur during the day and rest occurs during the night, the SCN amplitude will be negatively affected and shows a reduced amplitude. Horizontal lines indicate peak and trough SCN activity levels. The LD12:12 light cycle is indicated above the record (white, lights on; black, lights off).

While the current results are obtained from nocturnal animals, it would be interesting to know how behavioral activity affects the clock in diurnal animals. Notably, the circadian profiles of 5-HT are linked to the animal's 24 hrs activity-rest cycle rather than to the light-dark cycle, which implicates that these neuronal afferents cycle in anti-phase with the SCN in nocturnal animals, but is in phase in diurnal animals.[42] In humans, behavioral activity has a synchronizing effect on the circadian clock[8,19,43-45] and may have direct beneficial implications for health.[20,44] For instance, in the elderly and in people suffering from Alzheimer's disease or affective disorders, increased daytime exercise leads to improvement of sleep-wake rhythms, mood and performance.[20,45-49] Transgenic mice with disturbed behavioral activity patterns show an improvement of behavioral activity with scheduled exercise.[50] Our results indicate that exercise may act by enhancement of the amplitude of the central pacemaker itself rather than through downstream mechanisms. The enhancement of rhythm amplitude of the clock could have beneficial consequences for other rhythms that are under control of the SCN. People living against their clock, such as shift workers, may end up with reduced rhythm amplitude of the central clock, by being active at the reversed part of their cycle. This would consequently have adverse effects on other rhythms that are under control of the SCN. Properly scheduled exercise may thus have beneficial effects for a broad range of physiological functions.

Materials and methods

Ethics statement

All experiments were performed under the approval of the Animal Experiments Ethical Committee of the Leiden University Medical Centre. Adult male C57BL/6 mice (Harlan) received electrode implantation under anesthesia with analgesics. The animal was connected to the recording system with a counterbalanced swivel system enabling the animal to move freely during the measurements.

Micro-electrode implantations

Procedures for *in vivo* recordings of multiunit activity from SCN neurons have been described previously for mice.[51] At a minimum age of 12 wks (20–30 g), tripolar stainless steel micro-electrodes (Plastics One) were implanted in mice under Hypnorm/Dormicum anesthesia, using a stereotactic instrument. Two twisted electrodes (Polyimide-insulated; bare electrode diameter 0.125 mm) for differential recording were aimed at the SCN (coordinates: 0.46 mm posterior to bregma, 0.14 mm lateral to the midline, 5.2 mm ventral to the surface of the cortex, under a 5u angle in the coronal plane), and a third uncoated electrode was placed in the cortex for reference. The differential amplifier was based on the design of Yamazaki et al,[23] and allows for recordings also during the animals' behavioral activity.

SCN electrical activity recordings

After a recovery period of at least one week, the animal was individually housed in a temperature controlled recording chamber (22˚C), where food and water were available ad libitum and where the mouse could move freely. The electrical signal was amplified, bandwidth filtered, and window discriminators were used to convert action potentials into digital pulses that were counted in 2 sec bins (CircaV1.9 software, LUMC). Data were analyzed off-line by using OriginV7 software (OriginLab) and MATLAB (Mathworks Inc.).

Behavioral activity recordings

Recording chambers (floor size 35x36 cm) were equipped with a drinking sensor and a Passive Infra-Red (PIR) movement detector positioned 30 cm above the floor of the cage. The experimental set-up did not allow for the recording of running wheel activity. Behavioral activity profiles were recorded simultaneously with electrical activity in similar time bins (2 sec). The presence of activity as detected by the passive infrared detector is represented by a all-or-nothing modus (e.g. **Fig. 1, Fig. 2**), or by a graded scale (e.g. **Fig. 3, Fig. 4, Fig. 5**). After surgery, 14 animals were kept on LD12:12, and after several cycles the animals were released into constant darkness (DD) or constant light (LL). As a routine, the presence of a light-induced response in SCN electrical activity was tested by 5-min light exposure during the subjective night (Naturalite light bulb; 76 mW/cm^2 = 105 lux). Activity measurements

were verified and inspected at more detailed levels by video-records provided by an infrared-sensitive camera (AXIS 221 Network Camera, NL) that was placed in front of the cage. The cage was illuminated with infrared LEDs to provide a clear image for the infrared-sensitive camera in the dark. Type and intensity of spontaneous behavioral activity were analyzed off-line. In order to test the SCN responsiveness to 'forced' activity, behavioral activity was induced in 6 animals by mild disturbance of the animal's rest. For this test, we used only animals that showed suppressions of SCN impulse frequency with spontaneous behavioral activity. Procedures included either the production of some noise in the proximity of the animal's cage or small movements of the floor of the cage.

Data analysis – SCN electrical activity

Electrical activity data were smoothed in MATLAB (Mathworks Inc.). The smoothing parameter was set to the lowest value yielding a single peak and through per circadian cycle. Rhythm amplitudes were determined by measurement of the difference between peaks and troughs. The magnitude of the behaviorallyinduced neuronal suppression was defined as the difference between baseline electrical activity and the average electrical discharge during the suppression. The baseline level was defined as the mean discharge rate during the last 2 minutes before the suppression. The magnitude of the suppression was expressed as a percentage of the amplitude of the SCN rhythm. Single factor ANOVA analysis was performed to determine whether the electrical activity level during the animal's behavioral activity was significantly inhibited as compared to baseline level. The magnitude of the suppression was studied as a function of circadian time in 6 animals over a period of 3–7 cycles. Two-way ANOVA with post-hoc Bonferroni test was used to test for differences between day (CT0-12) and night (CT12–24) and $P < 0.05$ was considered to be statistically significant.

Data analysis – SCN subpopulation activity

To enable a more detailed analysis of SCN sub-population activity, the electrical signal was digitized at 25 kHz using Spike2 hardware and software (Cambridge Electronic Design). Off-line, the data were imported into MATLAB as 'waveform data' using parts of the sigTOOL SON Library (http://sigtool.sourceforge.net). A baseline recording (100 sec before behavioral activity occurred) was used to create a spike amplitude histogram. On the basis of this amplitude histogram, thresholds were set in such a way that the average number of counts within each threshold window was equal. Threshold windows were non-overlapping and started above noise level. Action potentials were counted within each step of set thresholds. The measurement of SCN subpopulations on the basis of baseline electrical activity levels leaves the neuronal response after baseline as a free parameter, and enables comparisons between different, approximately equal-sized, populations of neurons.

Video-analysis

By video-analysis, the following behavioral activities were scored in 1 sec bins: digging, drinking, eating, grooming, lying, moving, rearing, sitting, and walking. For the analysis, we used the data of 4 animals from which we had long recordings that included all types of scored behavior. With respect to the protocol for 'induced' behavioral activity, video was recorded to assure the induction of behavioral activity and to qualitatively analyze the type of behavioral activity following the mild disturbance procedure.

Histology

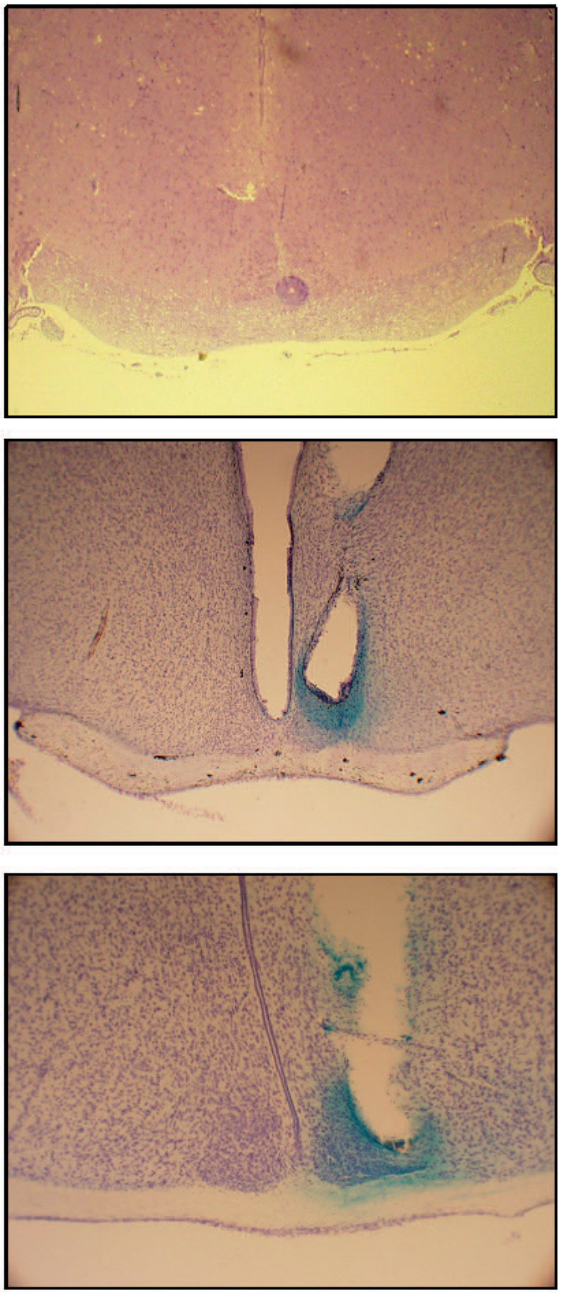
To verify the electrode location at the end of the experiment, animals received an overdose of barbiturate, after which a small electrolytic current was passed through the recording electrode to deposit iron at the electrode tip. The brains were collected and immersed in a potassium ferrocyanide containing fixative solution which caused the iron deposits in the brain to turn blue. Brains were sectioned coronally (10 mm slices) and stained with neutralred for microscopic reconstruction of the recording site (**Suppl. Fig. 2**). Recordings from outside the SCN were excluded from analysis.

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Suppl. Fig. 2. Histological sections containing the SCN and electrode track. Microscopic images of coronal brain sections from three mice showing the location of the electrode, which is marked by a blue spot and/or mechanical track damage. The suprachiasmatic nuclei are visible as clusters of densely stained cells, embedded in the optic chiasm at both sides of the third ventricle.

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PART II

Health outcomes of altered circadian rhythms

CHAPTER 7

The suprachiasmatic nucleus controls energy metabolism and insulin sensitivity

Claudia P. Coomans,[1,3,*] Sjoerd A.A. van den Berg,[2,*] Eliane A. Lucassen,[1] Thijs Houben,[1] Amanda C.M.Pronk,[2] Rianne D. van der Spek,[4] Andries Kalsbeek,[4] Nienke R. Biermasz,[3] Ko Willems van Dijk,[2] Johannes A. Romijn,[3,†] and Johanna H. Meijer[1,†]

*C.P.C. and S.A.A.v.d.B. contributed equally to this study.
[†]J.A.R. and J.H.M. contributed equally to this study.

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1. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
2. Department of Human Genetics, Leiden University Medical Center, Leiden, the Netherlands.
3. Department of Endocrinology and Metabolism, Academic Medical Center, Amsterdam, The Netherlands
4. Department of Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands.

Abstract

Disturbances in the circadian system are associated with the development of type 2 diabetes mellitus. Here, we studied the direct contribution of the suprachiasmatic nucleus (SCN), the central pacemaker in the circadian system, in the development of insulin resistance. Exclusive bilateral SCN lesions in male C57Bl/6J mice, as verified by immunochemistry, showed a small but significant increase in body weight (+17%), which was accounted for by an increase in fat mass. In contrast, mice with collateral damage to the ventromedial hypothalamus and paraventricular nucleus showed severe obesity and insulin resistance. Mice with exclusive SCN ablation revealed a loss of circadian rhythm in activity, oxygen consumption, and food intake. Hyperinsulinemic–euglycemic clamp analysis 8 wks after lesioning showed that the glucose infusion rate was significantly lower in SCN lesioned mice compared with sham-operated mice (-63%). Although insulin potently inhibited endogenous glucose production (-84%), this was greatly reduced in SCN lesioned mice (-7%), indicating severe hepatic insulin resistance. Our data show that SCN malfunctioning plays an important role in the disturbance of energy balance and suggest that an absence of central clock activity, in a genetically intact animal, may lead to the development of insulin resistance.

Introduction

Obesity and type 2 diabetes mellitus have an increasing prevalence in modern society. In the past decade, a strong and potentially causal relationship between metabolic disorders and disturbances of the circadian system has been elucidated.[1] The circadian system is responsible for 24 hr rhythms in a wide variety of physiological and behavioral functions.[2,3] Generation of these rhythms occurs in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus[2,3] and is explained by a transcriptional–translational feedback loop involving the clock genes CLOCK, BMAL1, Period (Per), and Cryptochrome (Cry).[1] The rhythms of the SCN are synchronized to the environmental 24 hr cycle mainly by light–dark information perceived by the eyes. Rhythmic information is transferred from the SCN to the central nervous system and to peripheral organs of the body.[4,5] This output is crucial for synchronization of many metabolic and endocrine factors such as glucose, insulin,[6,7] cortisol,[8] leptin, ghrelin,[9,10] and neuropeptide Y.[11]

Disturbances in circadian rhythms occur as a consequence of shift work and transitions in time zones, but also because of irregular sleep–wake patterns,[12] aging, or neurodegenerative disorders.[13] Animal studies have indicated a link between clock gene mutations and metabolic disturbances. For instance, Clock mutant mice have a greatly attenuated diurnal feeding rhythm, are hyperphagic and obese, and have development of a metabolic syndrome of hyperleptinemia, hyperlipidemia, hepatic steatosis, and hyperglycemia.[14] Because clock gene mutants are not specific to the SCN, the detrimental effects of disturbed rhythms may have their origin in peripheral organs other than the SCN. It is not clear to what extent the SCN itself is involved in metabolic disorders. Given the accumulating evidence for disturbances of SCN cellular organization in aging,[15] neurodegenerative disorders, and dementia,[13,16] this question is also clinically relevant, as it would explain comorbidity between various disorders.

To assess the role of disturbed function of the SCN *per se* in the development of obesity and type 2 diabetes mellitus, we performed bilateral microlesions of the SCN in male C57Bl/6J mice. Because the SCN is anatomically surrounded by areas regulating energy homeostasis, such as the ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN), great care was taken to distinguish between exclusively SCN lesioned mice and mice with collateral damage to surrounding nuclei. We show that selective ablation of the SCN results in complete loss of circadian energy metabolism and, moreover, in the development of severe hepatic insulin resistance, stressing the role of the central clock in the pathophysiology of insulin resistance.



Research design and methods

Animals

All animal experiments were approved by the Animal Ethic Committee from the Leiden University Medical Center (Leiden, the Netherlands). Male C57Bl/6J mice were housed individually in a controlled environment (21°C; 40–50% humidity) under a 12 hr/12 hr light/dark cycle (07:00–19:00 hrs) unless otherwise mentioned. Food (chow, RM3; Special Diet Services, Sussex, U.K.) and tap water were available ad libitum during the entire experiment. Body weight was monitored weekly for all individual mice.

SCN lesions

Bilateral ablation of the SCN was performed in 13-week-old mice as described previously.[17] Mice were anesthetized using a mixture of ketamine (100 mg/kg; Aescoket, Boxtel, the Netherlands), xylazine (10 mg/kg; Bayer AG, Leverkusen, Germany), and atropine (0.1 mg/kg; Pharmachemie, Haarlem, The Netherlands) and mounted in a stereotactic device (Digital Just for Mouse Stereotaxic Instrument; Stoelting, Wood Dale, IL). After identification of bregma, a hole was drilled through which the lesion electrode was inserted into the brain. Lesion needles were made by isolating a 0.3-mm stainless steel insect pin using isolating resin, except for 0.2 mm at the tip. The electrode tip was aimed at the SCN, 0.46 mm posterior to bregma, 0.15 mm lateral to the midline, and 5.2 mm ventral to the surface of the cortex.[21] Bilateral SCN lesions were made by passing a 0.6 mA current through the electrode for duration of 10sec. The sham lesioned mice underwent the same operation, but no current was passed through the electrode.

Circadian rhythm analysis

After SCN lesioning, all mice were housed in constant dark for 10 consecutive days to determine circadian rhythm in behavioral activity of each animal using passive infrared motion detection sensors (Hygrosens, Löffingen, Germany) that were mounted underneath the lid of the cage and connected to a ClockLab data collection system (Actimetrics) that recorded the amount of sensor activation in 1-min bins. The presence of circadian rhythms was determined by F-periodogram analysis based on the algorithm of Dörrscheidt and Beck.[18] Mice were included in the lesion group when no significant rhythm was present.

Positional check of the SCN lesion

The SCN lesions were checked as described previously.[10] To verify the position of the SCN lesions, brains were removed and fixed by immersion with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C. For cryoprotection, the brain tissue was equilibrated for 48 hrs with 30% sucrose in 0.1 mol/L Tris-buffered saline before sectioning. Thereafter, the brain tissue was cut into 30-mm sections and divided into two equal vials for

immunocytochemical staining. The two vials of brain sections were incubated overnight at 4°C with either rabbit antivasopressin or rabbit anti-vasoactive intestinal peptide primary antibodies. Sections were then rinsed in 0.1 mol/L Tris-buffered saline, incubated 1 hrs in biotinylated goat anti-rabbit IgG, and subsequently incubated for 1 hrs in avidin– biotin complex (Vector Laboratories, Burlingame, CA). The reaction product was visualized by incubation in 1% diaminobenzidine with 0.01% hydrogen peroxide for 5–7 min. Nickel ammonium sulfate (0.05%) was added to the diaminobenzidine solution to darken the reaction product (diaminobenzidine/ nickel). All sections were mounted on gelatin-coated glass slides, dried, run through ethanol and xylene, and covered for observation by light microscopy. For every animal, we blindly scored the amount of damage to the SCN and surrounding hypothalamic nuclei involved in metabolism (PVN and VMH).

Indirect calorimetry and metabolic cages

Individual measurements by indirect calorimetry were performed for a period of at least 4 consecutive days (Comprehensive Laboratory Animal Monitoring System; Columbus Instruments, Columbus, OH).[19] A period of 24 hrs was included at the start of the experiment to allow acclimatization of the mice to the cages. Experimental analysis started at 09:00. Analyzed parameters included real-time energy, water intake, and activity. Oxygen consumption (energy expenditure) measurements were performed at intervals of 7 min throughout the whole period. Oxygen consumption, activity, and energy intake were analyzed separately for day and night.

Hyperinsulinemic–euglycemic clamp analysis

Eight weeks after SCN lesioning, hyperinsulinemic–euglycemic clamp experiments were performed as previously described.[20] Mice were fasted for 16 hrs with food withdrawn at 17:00 the day before the study. All mice ate within 60 min before the start of the fasting period, minimizing differences in fasting time. During the experiment, mice were anesthetized by intraperitoneal injection with a combination of acepromazin (6.25 mg/kg; Sanofi Sant Nutrition Animale, Libourne Cedex, France), midazolam (6.25 mg/kg; Roche, Mijdrecht, the Netherlands), and fentanyl (0.31 mg/kg; Janssen-Cilag, Tilburg, the Netherlands). Anesthesia and body temperature were maintained throughout the procedure. At the end of the basal and the hyperinsulinemic periods, hematocrit values were determined to ensure that the mice were not anemic. First, basal rates of glucose turnover were determined by administering a primed (0.8 mCi) constant (0.02 mCi/min) intravenous infusion of 3-^{3}H-glucose (specific activity, 9.6 GBq/ mmol; Amersham, Little Chalfont, U.K.) for 60 min. Glucose rate of disposal (Rd; mmol/min/kg) was determined as the rate of tracer infusion (dpm/min) divided by the plasma-specific activity of 3-^{3}H-glucose (dpm/mmol) at both time points. Subsequently, insulin (actrapid; Novo Nordisk, Denmark)

was administered intravenously by primed (4.1 mU), constant (6.8 mU/h) infusion to attain steady-state circulating insulin levels of ~4 ng/mL together with 3-^{3}H-glucose. A variable intravenous infusion of a 12.5% D-glucose solution was used to maintain euglycemia as determined at 10-min intervals via tail bleeding (<3 mL; Accu-chek, Sensor Comfort; Roche Diagnostics). All mice were clamped at their respective basal glucose levels, because it has been shown that alterations in basal fasting glucose levels alone are sufficient to affect insulin sensitivity.[21] Seventy minutes after the start of the hyperinsulinemic period, when glucose infusion rates (GIRs) for all mice were stable for at least 30 min, blood samples were taken at 10-min intervals for 30 min to determine 3-^{3}H-glucose. Hyperinsulinemic Rd was determined similar to the basal period. The endogenous glucose production (EGP) was calculated as the difference between Rd and the GIR. After the hyperinsulinemic–euglycemic clamp, body composition (lean *vs.* fat mass) was determined by DEXA using the Norland pDEXA Sabre X-Ray Bone Densitometer (Norland, Hampshire, U.K.). All data were analyzed according to the software and recommendations of the manufacturer. Subsequently, the mice were euthanized.

Plasma analysis

Plasma insulin concentrations were measured by ELISA (Crystal Chem). For measurement of plasma 3-^{3}H-glucose, trichloroacetic acid (final concentration 2%) was added to 10 mL plasma to precipitate proteins using centrifugation. The supernatant was dried to remove water and was resuspended in milliQ. The samples were counted using scintillation counting (Packard Instruments).

Statistical analysis

Statistical analysis was performed using SPSS 17 for Windows (SPSS, Chicago, IL). Unpaired *t*-tests were performed for all comparisons, with statistical significance threshold set at $P = 0.05$.

Results

Behavioral and histological verification of SCN lesions.

Periodogram analysis of activity showed that all sham-operated mice retained a strong circadian rhythm (**Fig. 1A**), whereas SCN lesioned mice lost their circadian rhythm in activity after the lesion procedures (**Fig. 1B**). Histological analysis revealed SCN lesions without collateral damage in six mice. In addition to ablation of the SCN, four other mice had unilateral damage to the PVN, 10 other mice had bilateral damage to the PVN, and nine other mice had damage to both the PVN and the VMH. Sham-operated controls ($n = 17$) did not reveal damage to any of the aforementioned brain areas. Representative histological

sections of the hypothalamus, illustrating the histological verification of lesion position and size, are shown in **Suppl. Fig. 1**; 40-mm coronal sections were alternately stained for arginine vasopressin (immunohistochemical staining, **Suppl. Fig. 1A**), vasoactive intestinal peptide (immunohistochemical staining, **Suppl. Fig. 1B**), and cresyl violet (cell nuclei staining, **Suppl. Fig. 1C**).

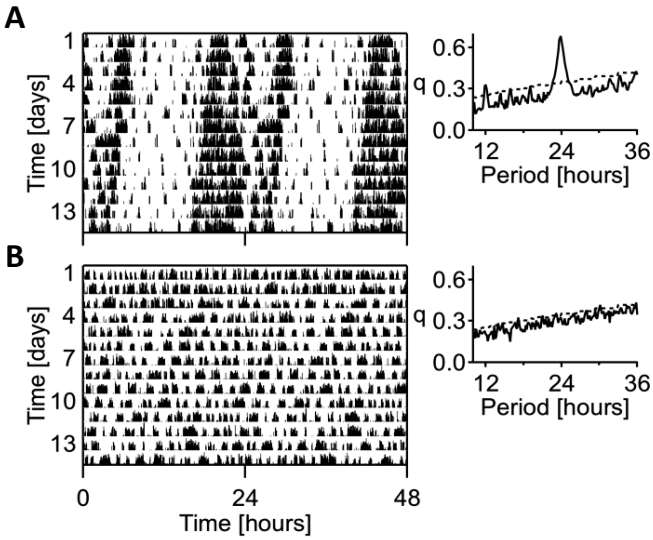
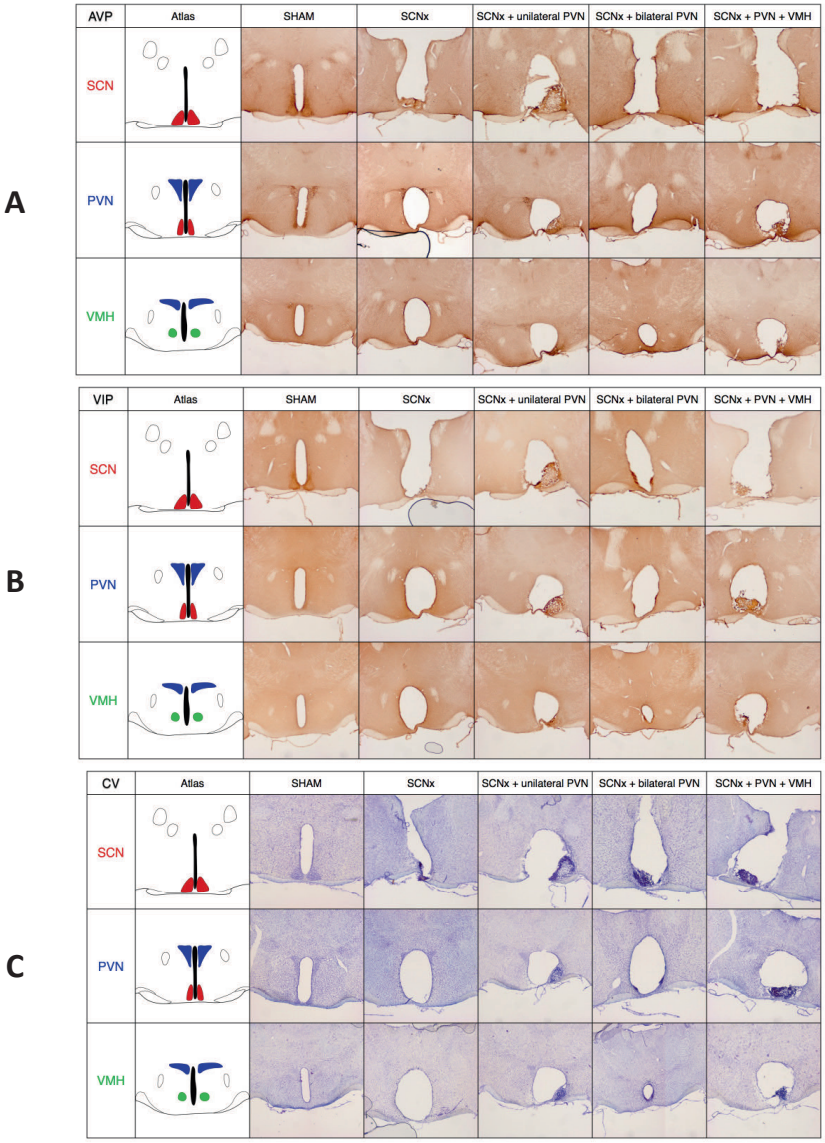


Fig. 1. Representative double-plotted actogram analyses of shamoperated (**A**) and SCN lesioned (**B**) mice under constant dark conditions. Each line of the double-plotted actograms represents 48hrs.

Selective SCN lesions

Body weight and composition

Before the operation and after a recovery period of 8 days, the average body weight of the sham and SCN lesioned mice did not differ (operation day: sham, 26.3 ± 1.5 g, SCN lesion, 26.0 ± 1.5 g, not significant; day 8: sham, 25.9 ± 1.6 g, SCN lesion, 25.3 ± 1.6 g, not significant). Eight weeks after SCN lesion, body mass of SCN lesioned mice was 17% higher compared with that of the sham mice (24.9 ± 1.2 *vs.* 29.2 ± 1.9 g; $P < 0.01$; **Fig. 2**). Assessment of body composition revealed that fat mass was significantly higher in SCN lesioned mice compared with sham (5.8 ± 2.6 *vs.* 1.9 ± 1.0 g; $P < 0.01$), whereas lean mass did not differ (17.7 ± 2.2 *vs.* 17.2 ± 1.7 g; not significant). This indicates that SCN ablation results in only mild overweight status compared with sham mice.



Suppl. Fig. 1. Representative histological sections of the hypothalamus, illustrating the histological verification of lesion position and size. 40 micrometer coronal sections were alternately stained for arginine vasopressin (AVP, immuno-histochemical staining, **A**), vasoactive intestinal peptide (VIP, immuno-histochemical staining, **B**) and cresyl violet (CV, cell-nuclei staining, **C**). For each animal, the location of lesion-induced damage was judged by independent researchers. Animals were classified based on (I) the presence of a bilateral ablation of the suprachiasmatic nucleus (SCN, top row of each panel) and for the presence of additional damage: (II) unilateral or bilateral ablation of the paraventricular nucleus (PVN, middle row in each panel) and (III) damage to the rostral part of the ventromedial hypothalamus (VMH, bottom row in each panel). For comparison, schematic drawings of the mouse hypothalamus (first column) and stainings of sham-operated animals (second column) are included.

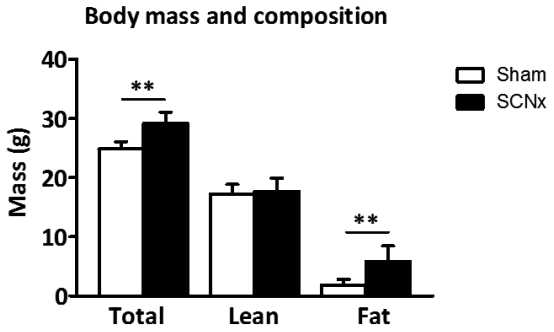


Fig. 2. Body mass and composition of sham (white bars) and SCN lesioned (black bars) mice at the time of the hyperinsulinemic–euglycemic clamp. Total body mass, lean body mass, and fat mass were determined. Data are represented as mean ± SD. $^{**}P < 0.01$.

Indirect calorimetry

Metabolic cage data on oxygen consumption, food intake, and activity were obtained over a period of a minimum of 4 consecutive days and were analyzed separately for day and night. During the night, oxygen consumption was higher compared with the day in sham mice (3,386 ± 174 *vs.* 2,992 ± 155 mL/kg/h; $P < 0.01$; **Fig. 3A**). This circadian rhythm in oxygen consumption was lost in SCN lesioned mice, resulting in higher oxygen consumption during the day in SCN lesioned mice compared with shammice (3,321 ± 252 *vs.* 2,992 ± 155 mL/kg/h; $P < 0.01$). Total 24 hr oxygen consumption was not different between sham and SCN lesioned mice (76,531 ± 3,854 *vs.* 79,518 ± 7,595 mL/kg/day, not significant). During the night, sham mice were more active compared with during the day (195 ± 68 *vs.* 79 ± 33 beam breaks (bb); $P < 0.01$; **Fig. 3B**). In line with the oxygen consumption rates, circadian pattern in activity was lost in SCN lesioned mice (123 ± 48 *vs.* 128 ± 38 bb, not significant). This loss in activity pattern in SCN lesioned mice resulted in increased activity during the day (128 ± 38 *vs.* 79 ± 33 bb; $P < 0.01$) and reduced activity during the night compared with sham mice (123 ± 48 *vs.* 195 ± 68 bb; $P < 0.01$). Total 24 hr activity, however, was not different between sham and SCN lesioned mice (273 ± 96 *vs.* 251 ± 81 bb; not significant). Sham mice consumed 68% of their total food during the night (night *vs.* day: 3.2 ± 0.5 *vs.* 1.5 ± 0.2 g; $P < 0.01$; **Fig. 3C**), whereas SCN lesioned mice consumed only 54% during the night (night *vs.* day: 1.9 ± 0.3 *vs.* 1.6 ± 0.9 g, not significant). This resulted in reduced food intake during the night for SCN lesioned mice compared with sham mice (1.9 ± 0.3 *vs.* 3.2 ± 0.5 g; $P < 0.01$). Furthermore, total 24 hr food intake also was reduced for SCN lesioned mice compared with sham mice (3.5 ± 1.2 *vs.* 4.7 ± 0.5 g; $P < 0.01$). Calculating the respiratory exchange ratio showed that SCN lesioned mice had a lower respiratory exchange ratio compared with sham mice during the day (0.86 ± 0.04 *vs.* 0.90 ± 0.01; $P < 0.05$) as well as during the night (0.87 ± 0.01 *vs.* 0.96 ± 0.02; $P < 0.01$). These data clearly show that ablation of the SCN results in a loss of circadian rhythm in respiratory metabolism and food intake.

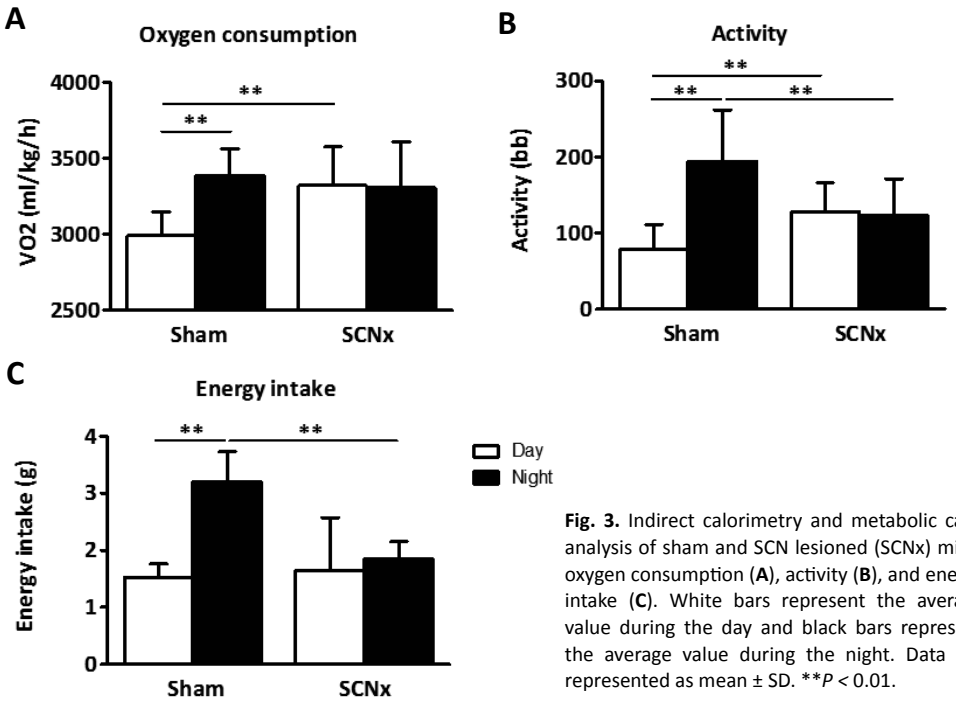


Fig. 3. Indirect calorimetry and metabolic cage analysis of sham and SCN lesioned (SCNx) mice: oxygen consumption (**A**), activity (**B**), and energy intake (**C**). White bars represent the average value during the day and black bars represent the average value during the night. Data are represented as mean ± SD. $^{**}P < 0.01$.

Hyperinsulinemic–euglycemic clamp studies

Hematocrit levels were similar at the basal and the hyperinsulinemic period for sham and SCN lesioned mice, indicating that the mice were not anemic. At the start of the clamp, insulin, glucose, and free fatty acid (FFA) plasma levels were significantly higher in SCN lesioned mice compared with sham controls (**Table 1**). During the hyperinsulinemic period, insulin levels were increased to a similar extent for both the sham and SCN lesioned mice. At the end of the hyperinsulinemic period, circulating glucose levels were isoglycemic compared with fasting levels in sham and SCN lesioned mice, resulting in significantly higher glucose levels in the hyperinsulinemic period for SCN lesioned mice compared with sham mice (**Fig. 4A**). At the end of the clamp, FFA levels were decreased compared with basal plasma levels in sham and SCN lesioned mice, although this failed to reach statistical significance in SCN lesioned mice ($P = 0.08$). GIRs over the final 20 min of the clamp were significantly lower in SCN lesioned mice compared with sham controls (27.6 ± 19.5 *vs.* 74.7 ± 21.1 mmol/min/kg; $P < 0.01$; **Fig. 4B and C**). The glucose-specific activities measured at 10-min intervals indicated the presence of steady-state conditions in all groups (**Suppl. Table 1**).

Table 1. Body mass and hyperinsulinemic–euglycemic clamp parameters of sham and suprachiasmatic nucleus lesioned (SCNx) mice.

	Sham		SCNx	
	Basal	**Clamp**	**Basal**	**Clamp**
Body mass (g)	24.9 ± 1.2		29.2 ± 1.9†	
Insulin (ng/ml)	0.5 ± 0.3	5.2 ± 1.1‡	1.0 ± 0.3†	4.8 ± 1.9‡
Glucose (mmol/l)	4.9 ± 0.8	4.7 ± 0.9	6.7 ± 1.2†	6.8 ± 1.2†
FFA (mmol/l)	0.9 ± 0.2	0.6 ± 0.3‡	1.2 ± 0.3*	0.8 ± 0.2
Hematocrit (%)	41.6 ± 4.7	38.5 ± 2.3	41.7 ± 2.7	40.5 ± 0.7

Data are represented as mean ± SD. *$P < 0.05$ *vs.* sham. †$P < 0.01$ *vs.* sham. ‡$P < 0.05$ basal vs hyperinsulinemic–euglycemic clamp period.

Suppl. Table 1. Glucose (**A**) and tracer specific activity (**B**) data for all groups during the basal and hyperinsulinemic period of the clamp. Data are represented as mean ± SD.

A

	Basal 1 [Glucose] (mmol/l)	**Basal 2 [Glucose] (mmol/l)**	**Hyper 1 [Glucose] (mmol/l)**	**Hyper 2 [Glucose] (mmol/l)**	**Hyper 3 [Glucose] (mmol/l)**
Sham	5.0 ± 0.7	4.9 ± 1.0	4.6 ± 0.8	4.7 ± 1.0	4.7 ± 1.0
SCNx	6.6 ± 1.1	6.8 ± 1.4	7.4 ± 1.3	6.7 ± 1.1	6.8 ± 1.1
SCNx + unilat PVN damage	6.7 ± 0.6	7.3 ± 2.0	7.0 ± 1.1	7.1 ± 1.2	7.1 ± 1.2
SCNx + bilat PVN damage	7.2 ± 0.9	7.4 ± 1.5	7.2 ± 0.7	7.2 ± 0.9	7.2 ± 1.3
SCNx + PVN/VMH damage	7.6 ± 1.7	7.6 ± 1.9	6.8 ±1.0	7.4 ± 1.7	7.4 ± 1.6

B

	Basal 1 SA (DPM/µmol)	**Basal 2 SA (DPM/µmol)**	**Hyper 1 SA (DPM/µmol)**	**Hyper 2 SA (DPM/µmol)**	**Hyper 3 SA (DPM/µmol)**
Sham	12100 ± 3966	12855 ± 4176	7831 ± 2283	7519 ± 2587	7398 ± 1990
SCNx	8849 ± 1828	8891 ± 1803	6066 ± 283	5452 ± 1044	6659 ± 1241
SCNx + unilat PVN damage	9925 ± 2274	9383 ± 2606	6842 ± 1804	7085± 1365	7109 ± 2013
SCNx + bilat PVN damage	6450 ± 1098	6887 ± 1864	5775 ± 1339	6031 ± 1625	6755 ± 2227
SCNx + PVN/VMH damage	7532 ± 4189	8174 ± 5039	5302 ± 1906	6068 ± 2177	5920 ± 2677

In the basal period, EGP, which equals Rd, was not different between sham and SCN lesioned mice (53.6 ± 16.6 *vs.* 65.0 6 8.6 mmol/min/kg, not significant; **Fig. 5A**). In the hyperinsulinemic–euglycemic period, Rd was increasedby 57% in the sham mice compared with the basal period (83.9 ± 23.8 *vs.* 53.6 ± 16.6 mmol/min/kg; $P < 0.01$) and by 27% in the SCN lesioned mice, although this failed to reach statistical significance (82.8 ± 15.9 *vs.* 65.0 ± 8.6 mmol/min/kg; $P = 0.06$). Hyperinsulinemic Rd was not significantly different between sham and SCN lesioned mice. In the hyperinsulinemic–euglycemic period, EGP was decreased by 84% in sham-operated mice compared with the basal period (8.8 ± 12.2 *vs.* 53.6 ± 16.6 mmol/min/kg; $P < 0.01$; **Fig. 5B**), whereas EGP was decreased by only 7% in SCN lesioned mice (60.3 ± 25.8 *vs.* 65.0 ± 8.6 mmol/min/ kg, not significant). These data indicate that bilateral ablation of the SCN induces severe hepatic insulin resistance.

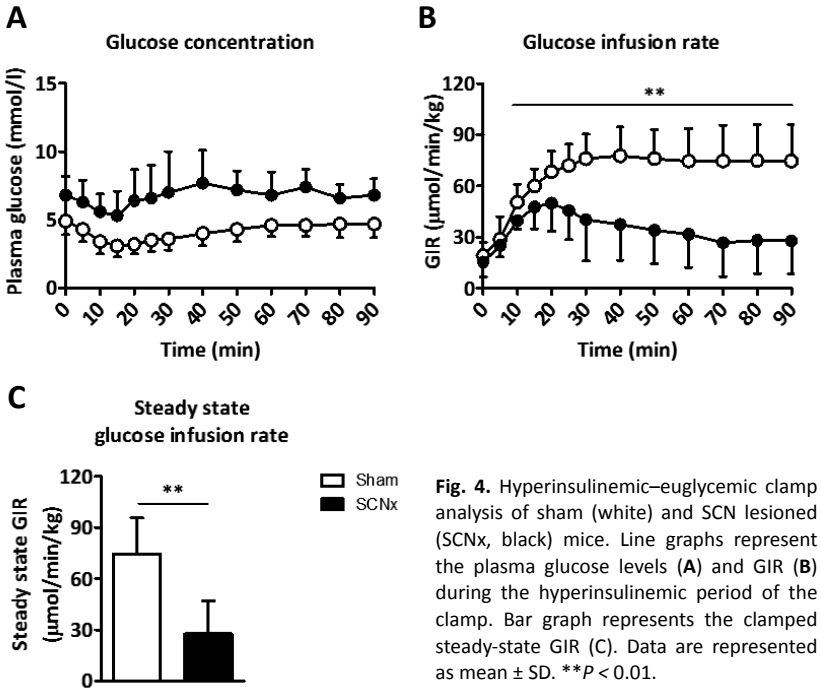


Fig. 4. Hyperinsulinemic–euglycemic clamp analysis of sham (white) and SCN lesioned (SCNx, black) mice. Line graphs represent the plasma glucose levels (**A**) and GIR (**B**) during the hyperinsulinemic period of the clamp. Bar graph represents the clamped steady-state GIR (C). Data are represented as mean ± SD. $^{**}P < 0.01$.

SCN lesion with collateral hypothalamic damage

SCN lesioned mice with collateral damage to hypothalamic nuclei involved in metabolism were analyzed separately. SCN lesioned mice with additional, but selective, unilateral damage to the PVN had modestly increased body mass compared with sham lesioned mice (29.9 ± 3.6 *vs.* 24.9 ± 1.2 g; $P < 0.01$; **Fig. 6A**), which was the result of increased fat mass (7.9 ± 2.5 *vs.* 1.9 ± 1.0 g; $P < 0.01$), but not lean mass (16.2 ± 1.0 *vs.* 17.2 ± 1.7 g, not significant).

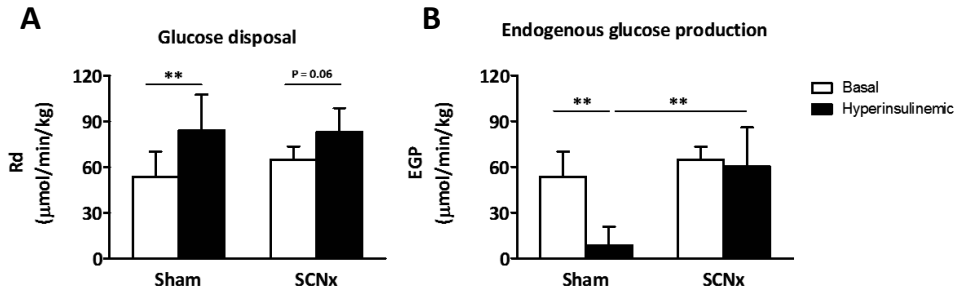


Fig. 5. Glucose Rd (**A**) and EGP (**B**) in basal period (white bars) and hyperinsulinemic–euglycemic clamp period (black bars) of sham and SCN lesioned (SCNx) mice. Data are represented as mean ± SD. $^{**}P < 0.01$.

SCN lesioned mice with bilateral damage to the PVN were considerably heavier than the sham mice (42.5 ± 3.3 *vs.* 24.9 ± 1.2 g; $P < 0.01$), which was the result of increased fat mass (21.3 ± 4.8 *vs.* 1.9 ± 1.0 g; $P < 0.01$), but this also coincided with a decrease in lean mass (13.6 ± 2.2 *vs.* 17.2 ± 1.7 g; $P < 0.01$). SCN lesioned mice with collateral damage to both the PVN and the VMH had development of extreme obesity compared with sham mice (49.3 ± 5.1 *vs.* 24.9 ± 1.2 g; $P < 0.01$), which was the result of an excessive increase in fat mass (24.7 ± 3.6 *vs.* 1.9 ± 1.0 g; $P < 0.01$), whereas lean mass was not different (16.8 ± 3.3 *vs.* 17.2 ± 1.7 g, not significant). Insulin sensitivity in SCN lesioned mice with collateral damage was determined by hyperinsulinemic–euglycemic clamp analysis. GIR needed to maintain euglycemia, as determined over a period of 20 min at stable plasma glucose values, was significantly lower in all SCN lesioned mice with collateral damage (SCN lesion + unilateral PVN damage: 41.3 ±11.0 mmol/min/kg; SCN lesion + bilateral PVN damage: 23.9 ± 13.1 mmol/min/kg; SCN lesion + PVN/VMH damage: 15.4 ± 13.5 mmol/min/kg) compared with sham controls (74.7 ± 21.1 mmol/min/kg; **Fig. 6B**), suggesting insulin resistance. Indirect calorimetry, metabolic cage, EGP, and Rd data and insulin levels of SCN lesioned mice with collateral damage are shown in **Suppl. Table 2**.

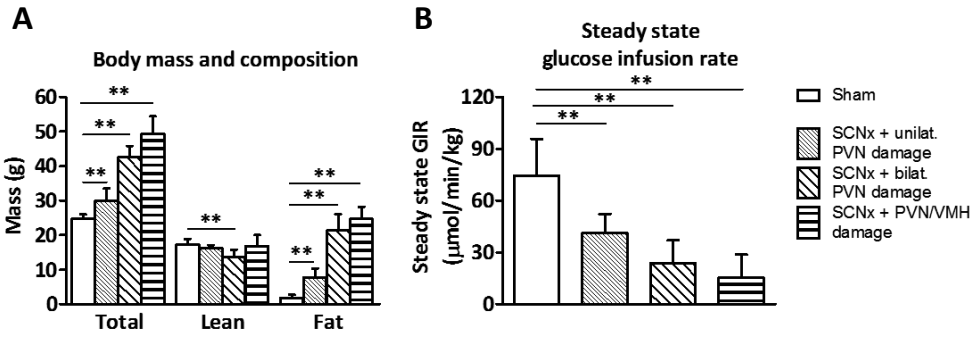


Fig. 6. Body mass and composition (**A**) and hyperinsulinemic–euglycemic clamp analysis (**B**) of sham (white bars) and SCN lesioned (SCNx) mice with collateral damage (striped bars): unilateral PVN, bilateral PVN, and PVN plus VMH. Data are represented as mean ± SD. $^{**}P < 0.01$.

Suppl. Table 2. Indirect calorimetry/metabolic cage (**A**), hyperinsulinemic-euglycemic clamp (**B**) and plasma insulin data (**C**) of sham and SCN lesioned (SCNx) mice with collateral damage. Data are represented as mean ± SD, **P* < 0.05 *vs.* sham, ***P* < 0.01 *vs.* sham, $^{\#}P$ < 0.05 light *vs.* dark, $^{\$}P$ < 0.05 basal *vs.* hyperinsulinemic-euglycemic clamp period.

A

	Dark		
	VO_2 (ml/kg/h)	Activity (bb/12 h)	FI (g/12 h)
Sham	3386 ± 173	195 ± 67	3.20 ± 0.53
SCNx + unilat PVN damage	3107 ± 162*	74 ± 22*	2.24 ± 0.54*
SCNx + bilat PVN damage	2530 ± 196*	61 ± 23*	2.52 ± 0.73*
SCNx + PVN/VMH damage	2594 ± 513*	57 ± 29*	2.42 ± 0.72*
	Light		
	VO_2 (ml/kg/h)	Activity (bb/12 h)	FI (g/12 h)
Sham	2992 ± 154$^{\#}$	79 ± 32$^{\#}$	1.53 ± 0.23$^{\#}$
SCNx + unilat PVN damage	3130 ± 200	92 ± 41	2.27 ± 0.92*
SCNx + bilat PVN damage	2600 ± 167*	93 ± 34$^{\#}$	1.60 ± 0.38
SCNx + PVN/VMH damage	2579 ± 495*	73 ± 45	2.27 ± 0.55*
	24 h		
	VO2 (ml/kg)	Activity (bb)	FI (g)
Sham	76531 ± 3831	273 ± 123	4.73 ± 0.50
SCNx + unilat PVN damage	74849 ± 4302	166 ± 62*	4.51 ± 1.43
SCNx + bilat PVN damage	61561 ± 4327*	154 ± 55*	4.13 ± 1.06
SCNx + PVN/VMH damage	62075 ± 12070*	131 ± 73*	4.69 ± 0.84

B

	Basal Rd (µmol/min/kg)	Hyperinsulinemic Rd (µmol/min/kg)	Hyperinsulinemic EGP (µmol/min/kg)
Sham	53.6 ± 16.6	83.9 ± 23.8	8.8 ± 12.2
SCNx + unilat PVN damage	61.1 ± 17.9	77.5 ± 31.6	36.2 ± 29.3*
SCNx + bilat PVN damage	61.0 ± 9.5	61.7 ± 21.1*	37.8 ± 18.3*
SCNx + PVN/VMH damage	51.2 ± 24.4	55.6 ± 19.8*	40.2 ± 23.1*

C

	Insulin (ng/ml)	
	Basal	Hyperinsulinemic
Sham	0.5 ± 0.3	5.2 ± 1.1$^{\$}$
SCNx + unilat PVN damage	0.8 ± 0.3	4.8 ± 1.1$^{\$}$
SCNx + bilat PVN damage	2.9 ± 1.5**	8.6 ± 2.4$^{\$}$**
SCNx + PVN/VMH damage	1.4 ± 0.6**	4.6 ± 1.7$^{\$}$

Discussion

This study addressed the effects of thermic, bilateral ablation of the SCN on energy metabolism and insulin sensitivity in mice. We show that exclusive lesions of the SCN disrupt the circadian pattern of energy intake, activity, and energy expenditure, as expected. However, although selective SCN ablation resulted in only mild overweight status compared with sham mice, hepatic insulin sensitivity was severely impaired. Therefore, disturbed SCN function has profound metabolic effects.

Anatomically, the SCN is connected with the VMH and the PVN through the subparaventricular zone of the hypothalamus.[22] In line with previous studies, mice with collateral damage to the PVN or VMH developed severe obesity and insulin resistance.[23-28] Interestingly, unilateral PVN damage in SCN lesioned mice did not result in additional overweight status, indicating that PVN damage results in obesity only in the case of bilateral damage. In light of our data, it is of utmost importance to ascertain correct and exclusive lesions of the SCN to study the role of the circadian pacemaker in the context of metabolism. However, the low success rate (~30%) of the operation in a previous study[10] and this study (~20%) limits the potential of this method to study the role of the SCN in metabolism, increasing the value of the current study.

The mild increase in weight gain in mice with exclusive SCN lesions compared with sham mice (+17%) is incontrast to a previous finding in rats.[10] In rats, lesions of the SCN did not induce an increase in body mass, whereas the effect on body composition was not determined. This discrepancy may be attributable to the use of the wellstudied obesogenic C57Bl/6J mouse strain[29-31] in our study, whereas the rat study was performed in Wistar rats. Wistar rats only become obese on hypercaloric food intake.[32]

Previously, it has been shown that the SCN is involved in the regulation of energy homeostasis in mice[33] and rats.[34] In the current study, indirect calorimetry and metabolic cage analysis revealed that ablation of the SCN induced a loss of circadian rhythm in oxygen consumption and activity without affecting the 24 hr average levels. This loss of circadian rhythm in homeostasis is in line with previous findings, in which SCN lesions eliminated a wide range of rhythms, including leptin.[10] Although total food intake over a period of 1 day and 1 night was reduced by 26%, SCN lesioned mice consumed more during the light part of the day compared with sham mice (46% *vs.* 32% of total food intake). Recently, it has been shown that mice and rats fed only during the day gained significantly more weight than mice fed only at night.[35-37] In these studies, obesity resulted from dissociation between the timing of food intake and the intrinsic rhythm of energy expenditure and, thus, animals were eating "against their clock time." In our study, the protocol was essentially different and animals were not forced to eat at the other part of the cycle, but rather their circadian system was impaired. Therefore, it remains unclear how the mice with selective SCN lesions developed mild overweight because they ate less than the controls and showed the same level of overall activity. Interestingly, results comparable with ours were found in continuous light–exposed mice.[38] Continuous light is a strong disruptor of SCN synchrony. When applied for prolonged periods of time, mice become arrhythmic. In continuous light–induced arrhythmic mice, total activity and food intake levels did not differ from that of mice in light/dark. Even though energy balance over 24 hrs was similar, mice exposed to continuous light displayed impaired glucose tolerance. It has been shown that the SCN exerts excitatory effects on thermogenesis by brown adipose tissue,[39] and the mild weight

gain in mice with a selective SCN lesion could be the result of reduced brown adipose tissue activity. Alternatively, changes in time distribution of food intake may play a role, which remains an interesting topic for future studies.

Indirect calorimetry and metabolic cage analysis further revealed that SCN lesioned mice had lower respiratory exchange ratio during the day and during the night as compared with sham mice. This shows that SCN ablation results in lower relative carbohydrate oxidation rates and, conversely, higher fat oxidation rates compared with sham mice. These data suggest that the oxidative response to food intake is less directed to carbohydrate metabolism is lesioned, a hallmark for an impaired metabolic flexibility.[40] Impaired metabolic flexibility has been associated with impaired insulin sensitivity in humans.[41]

Compared with the sham-operated mice, SCN lesioned mice were hyperglycemic and hyperinsulinemic in the postabsorptive state. Furthermore, SCN lesioned mice had increased fasting FFA levels, suggesting a possible removal of inhibitory input from the SCN to the adipose tissue, thereby increasing the basal rate of lipolysis. Increased circulating levels of FFA have been implicated as a possible pathway for development of insulin resistance in obesity.[42] Insulin sensitivity shows a circadian pattern in humans[43] as well as in rodents,[44] with insulin sensitivity being highest during the active period and lowest during the resting period. The protocol used in this study was such that the sham mice were clamped during their resting period, when insulin sensitivity is lowest, opting for conservative conclusions. Severe hepatic insulin resistance, but not peripheral insulin resistance, was present even though body fat mass was increased only minimally. The SCN is crucial for the circadian control of glucose production and glucose uptake.[45-48] Furthermore, there is a direct control of hepatic glucose metabolism resulting from cross-communication of the SCN and PVN, further mediated by innervation of the liver.[49] Therefore, it is likely that the impaired hepatic insulin sensitivity found in the SCN lesioned mice is, at least in part, a direct result of the disrupted SCN-mediated control of glucose and FFA metabolism.

All SCN lesioned mice with collateral damage to PVN or VMH had development of hepatic insulin resistance. Furthermore, SCN lesioned mice with bilateral PVN damage and SCN lesioned mice with PVN or VMH damage showed peripheral insulin resistance, most likely as a result of increased fat mass in these mice.

In conclusion, we demonstrate that exclusive deletion of the SCN induces loss in circadian rhythms in energy metabolism and food intake. Although ablation of the SCN resulted in only mild overweight status, SCN lesioned mice were severely insulin resistant in the liver. Great care was taken to distinguish between exclusive SCN lesioned mice from mice that had collateral damage to the PVN and VMH areas, because the latter resulted in severe obesity. It was previously shown that mice with mutations in clock genes have altered energy homeostasis and glucose metabolism.[14,50] Together, the data from several studies provide solid evidence that the SCN is crucially involved in the maintenance of energy balance and hepatic insulin sensitivity.

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CHAPTER 8

Environmental 24-hour cycles are essential for health

Eliane A. Lucassen,[1] Claudia P. Coomans,[1] Maaike van Putten,[2] Suzanne R. de Kreij,[1] Jasper H.L.T. van Genugten,[1] Robbert P.M. Sutorius,[1] Karien E. de Rooij,[3,6] Martijn van der Velde,[3] Sanne L.Verhoeve,[1] Jan W.A. Smit,[5] Clemens W.G.M. Löwik,[3] Hermelijn H. Smits,[4] Bruno Guigas,[4,7] Annemieke M. Aartsma-Rus,[2] Johanna H. Meijer[1]

Submitted.

1. Laboratory for Neurophysiology, Department of Molecular Cellular Biology, Leiden University Medical Center, the Netherlands.
2. Department of Human Genetics, Leiden University Medical Center, the Netherlands.
3. Department of Radiology, Leiden University Medical Center, the Netherlands.
4. Department of Parasitology, Leiden University Medical Center, the Netherlands.
5. Department of Medicine, Division of Endocrinology, Radboud University Medical Center, the Netherlands.
6. Percuros BV, the Netherlands.
7. Deptartment of Molecular Cellular Biology, Leiden University Medical Center, the Netherlands.

"Secundum naturam vivere" - Seneca, Letters to Lucilius, letter 5

Abstract

Circadian rhythms are deeply rooted in the biology of virtually all organisms. The pervasive use of artificial lighting in modern society disrupts circadian rhythms and can be detrimental to our health. To investigate the relationship between disrupting circadian rhythmicity and disease, we exposed mice to continuous light (LL) for 24 weeks and measured several major health parameters. Long-term neuronal recordings revealed that 24 weeks of LL reduced rhythmicity in the central circadian pacemaker of the suprachiasmatic nuclei (SCN) by 70%. Strikingly, LL exposure also reduced skeletal muscle function (forelimb grip strength, wire-hanging duration, and grid-hanging duration), caused trabecular bone deterioration, and induced a transient pro-inflammatory state. After the mice were returned to a standard light-dark cycle, the SCN neurons rapidly recovered their normal high-amplitude rhythm, and the aforementioned health parameters returned to normal. These findings strongly suggest that a disrupted circadian rhythm reversibly induces detrimental effects on multiple biological processes.

Introduction

Virtually all organisms have measurable circadian rhythms that help them anticipate and adapt to the environmental day-night cycle. In mammals, these circadian rhythms are orchestrated by neurons within the suprachiasmatic nucleus (SCN), which is located in the anterior hypothalamus. The SCN conveys temporal information to peripheral tissue oscillators, thus producing synchronized circadian rhythms in many bodily processes, including muscle function, bone metabolism, and immune system function.[1-4] Under evolutionary pressure, the circadian system evolved as a robust mechanism for adapting to life in a cyclic environment. Thus, we hypothesize that organisms require clear external cycles in order to maintain a healthy state and that absence of external rhythmicity is detrimental for health.

The use of artificial lighting in modern society—particularly during the night—disrupts the natural robust environmental cycle and is a risk factor for frailty.[5] Nowadays, 75% of the world's population is exposed to light during the night.[6] Moreover, the prevalence of shift work is relatively high around the globe; approximately 20% of workers in Europe, 29% of Americans, and 36% of Chinese and Koreans are engaged in shift work.[7-8] Importantly, epidemiological studies of shift workers revealed increased prevalence of breast cancer,[9] metabolic syndrome,[10] osteoporosis,[11] and bone fractures[12] in this population. In addition, individuals who are exposed to more light at night tend to have decreased sleep quality,[13] increased body weight,[14] and a higher prevalence of cardiovascular disease.[15] Although these studies suggest a correlation between artificial light exposure and health, they cannot determine whether this relationship is causal. Animal studies have shown that aberrant light exposure can affect both the immune system[16-18] and metabolic function.[19-20] However, in these studies, the exposure to light was relatively brief; therefore, the results cannot be translated directly to humans, who are often chronically exposed to disruptions in circadian rhythm.



To test whether long-term exposure to an aberrant light-dark cycle affects these health parameters, and to test whether these effects are reversible, we exposed mice to continuous light (LL) for 24 weeks, followed by 24 weeks in a standard light-dark (LD) cycle. To measure rhythmicity in the central clock, we performed *in vivo* electrophysiological recordings in the SCN of freely moving mice implanted with stationary electrodes. Although short-term exposure to LL has been reported to reduce SCN rhythmicity,[21-23] whether the effects of long-term LL exposure are chronic has not been studied previously. This issue is particularly important, as the SCN is highly plastic and can adapt to changes in photoperiod, even after exposure for three weeks or longer.[24] We measured the effect of long-term LL on skeletal muscle function, bone microstructure, and immune system function at various time points during and following 24 weeks of LL exposure. Our results support the hypothesis that long-term exposure to LL conditions has significant detrimental effects on a wide range of

relevant health parameters; moreover, the majority of these parameters rapidly returned to normal upon restoring the LD cycle. Thus, our results provide compelling evidence that an absence of environmental rhythmicity plays a causal role in susceptibility to disease.

Results

Reduced behavioral rhythms in continuous light

Wild-type mice (*n* = 134) were exposed to continuous light (LL) for 24 weeks ("experimental phase"), followed by a 12h:12h light-dark (LD) cycle for 24 weeks ("recovery phase"); as a control group, a separate set of age-matched mice (*n* = 119) were exposed to an LD cycle for the entire 48 weeks (**Fig. 1**). Although the strength of the circadian rhythm decreased with age in both the LL and LD groups, this effect was significantly greater in the LL mice compared to the control mice (**Fig. 1A-B**). At 2, 8, 16, and 24 weeks, the behavioral rhythm in the mice in the LL group was 19%, 36%, 50%, and 44% smaller, respectively, compared to the control group. Two and 8 weeks after returning to the light-dark cycle, rhythm strength had increased by 79% ($P < 0.001$) and 104% ($P < 0.001$), respectively, compared to rhythm strength at 24 weeks in LL. The period of behavioral rhythm was approximately 25.5 hours in the LL group, did not change over time, and recovered to 24 hours during the recovery phase (**Fig. 1C**). Mice in the LL group had a slight but significant decrease in activity after 2 weeks in continuous light; however, activity levels did not differ significantly between the two groups at any other time point (**Fig. 1D**). Upon returning to a standard light-dark cycle, the mice in the LL group rapidly recovered in terms of both rhythm period and rhythm strength (analyzed using the moving averages method; **Fig. 1E-F**). With respect to metabolism, the mice in the LL group were significantly heavier than age-matched mice in the control group, weighing an average of 2.8 and 2.4 g more at 8 and 24 weeks, respectively. Similarly, unfasted glucose levels were generally higher in the mice in the LL group throughout of the experimental phase, and this difference reached significance at 24 weeks (**Fig. 1H**). Strikingly, both the differences in weight and glucose levels disappeared after the LL mice were returned to a standard LD cycle (**Fig. 1G-H**).

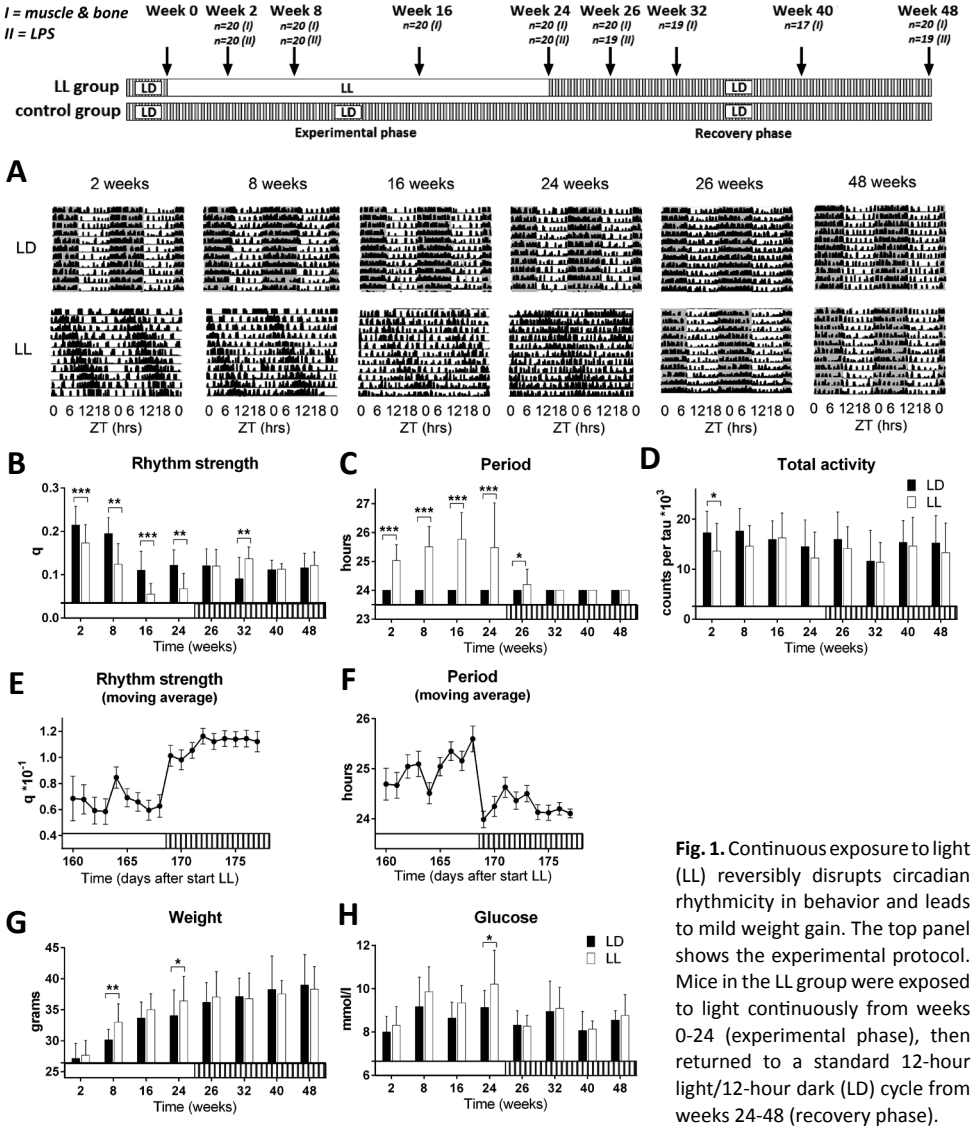


Fig. 1. Continuous exposure to light (LL) reversibly disrupts circadian rhythmicity in behavior and leads to mild weight gain. The top panel shows the experimental protocol. Mice in the LL group were exposed to light continuously from weeks 0-24 (experimental phase), then returned to a standard 12-hour light/12-hour dark (LD) cycle from weeks 24-48 (recovery phase). Mice in the control group were maintained under the standard LD cycle for the entire 48 weeks. At the indicated time points, mice were sacrificed for muscle and bone experiments or for LPS experiments. Mice used for the *in vivo* recordings are not included in the figure. (**A**) Examples of actograms recorded in LL and control (LD) mice at the indicated time points. Each horizontal row represents behavioral activity measured using a passive infrared motion detector on a double-plotted 24-hr day. Gray background represents the dark period, and white background represents the light period. (**B-D**) Rhythm strength, rhythm period, and total activity of mice in the LL and control groups at the indicated times (mean ± SD; *n* = 8-22 mice per group). (**E-F**) Five-day moving averages of rhythm strength and period in behavior in the final days of LL (or control) and in the first days after returning to an LD cycle (mean ± SEM; *n* = 22 per group). (**G-H**) Body weight and unfasted glucose levels in the LL and control groups at the indicated times (mean ± SD; *n* = 8-10 per group). The data were analyzed using a two-way ANOVA followed by post-hoc LSD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Continuous light exposure attenuates neuronal rhythms in the central clock

At t = 0 (i.e., baseline), the MUA recordings revealed high-amplitude rhythms, with higher levels of electrical activity during the subjective day than during the subjective night (**Fig. 2A**). When exposed to continuous light, this amplitude decreased initially to 63% of the baseline amplitude (**Fig. 2A-B**); at 8 and 24 weeks, the amplitude was reduced further to 34% and 30% of baseline, respectively. The strength of the behavioral rhythm was strongly correlated with MUA amplitude (R^2 = 0.754, P < 0.001, Pearson correlation), and fluctuations in the strength of the SCN rhythm within individual animals occurred in parallel with fluctuations in the strength of the animal's behavioral activity rhythm. These changes in the SCN's rhythm amplitude recovered rapidly upon shifting back to a standard light-dark cycle; importantly, this recovery was mediated primarily by a reduction in the SCN's firing rate in the dark period. Proper positioning of the microelectrode in the SCN was confirmed histologically (**Fig. 2C**, **Suppl. Fig. 1**).

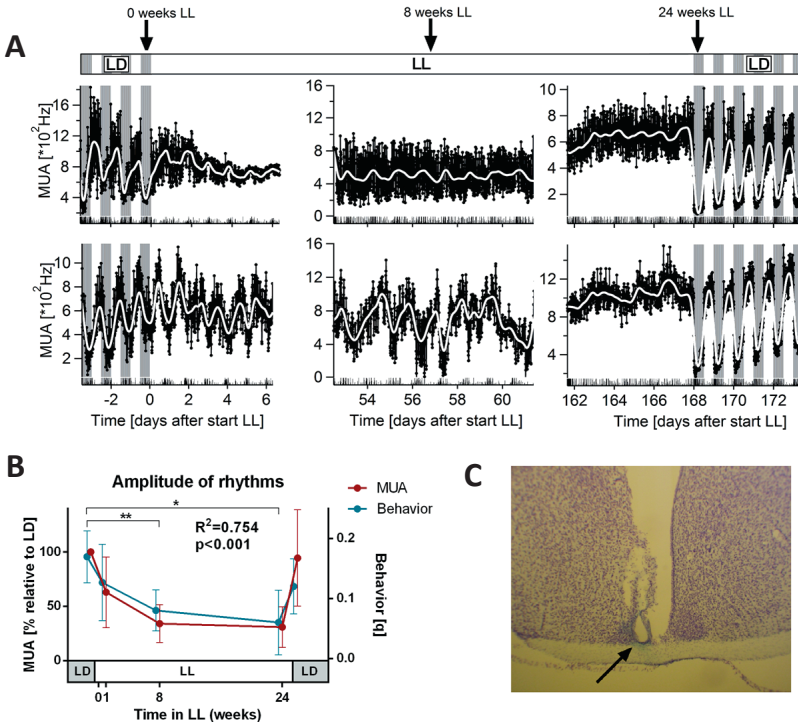
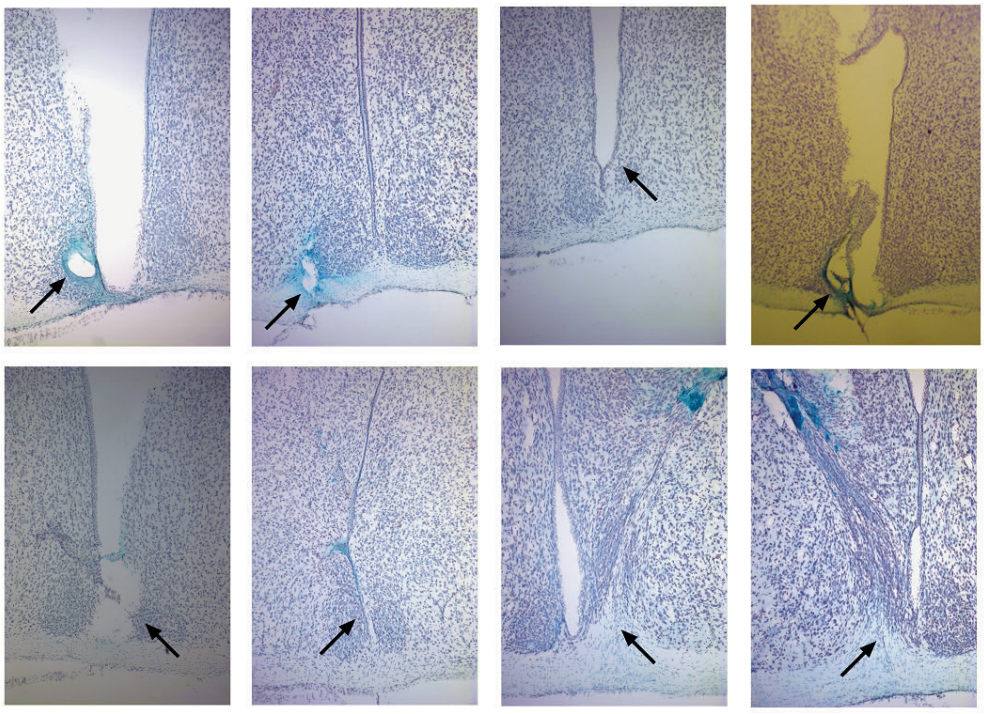


Fig. 2. Continuous exposure to light attenuates rhythmic neuronal activity in the central clock. *In vivo* recording electrodes were implanted in the SCN, and multiunit activity (MUA) was recorded in freely moving mice. (**A**) Examples of neuronal MUA rhythms recorded in the SCN of two LL mice at the times indicated. Note the more severe loss of rhythmicity in the mouse in the upper row. Gray bars indicate darkness. Behavioral activity is depicted as vertical upticks at the bottom of each graph. (**B**) Summary of the amplitude of the neuronal rhythm relative to the amplitude at baseline (red) and the strength of the behavioral rhythm of the same mice (blue). (**C**) Example of SCN histology with cresyl violet staining. The arrow indicates the location of the electrode. The third ventricle separates the two SCN nuclei that are embedded in the optic chiasm. Pearson correlation and repeated measures ANOVA, followed by post-hoc LSD; *P < 0.05, **P < 0.01.



Suppl. Fig. 1: Additional histological examples showing proper placement of the electrode tip in the SCN. In each image, the arrow indicates the location of the electrode tip. The samples were stained with cresyl violet and treated with ferrocyanide, which causes a blue color reaction with the iron deposited from the electrode tip. The third ventricle separates the left and right SCN, which are embedded in the optic chiasm.

Skeletal muscle function declines in animals exposed to continuous light

Performance in all three functional muscle tests declined over time in both groups of mice. During the experimental phase, the mice in the LL group performed significantly worse in grip strength and grid hanging duration at every time point compared to the mice in the control group; performance in the wire hanging test was also worse in the LL mice, with significantly different values measured at 8 weeks (**Fig. 3A-C**). The difference in skeletal muscle function between the two groups disappeared in the recovery phase. Additional analyses in which the results were corrected for body weight and behavioral activity yielded similar results.

Both groups of mice exhibited increased fatigue-related behavior (e.g., distance walked, behavioral intensity, and time spent rearing) and anxiety-related behavior (e.g., time spent in a corner) after completing the tests as analyzed by video analysis; however, these measures did not differ significantly between the LL and control groups (**Suppl. Fig. 2**). These observations suggest that the effect of continuous light exposure cannot be attributed to a difference in motivation or anxiety.

We found no difference between the two groups with respect to absolute CK levels, the pre-test/post-test CK ratio, or the change in CK levels, indicating that LL exposure did not induce muscle damage. Although the relative amount of fibrosis in the quadriceps muscle was significantly higher in LL mice compared to control mice at 24 weeks (percentage of collagen 5 ± 1% *vs.* 4 ± 1%, respectively), this difference was too small to account for the observed differences in muscle function. We found no significant difference in the mRNA levels of macrophage (*Lgals3*, *CD68*), fibrosis (*Col1α*), regeneration (*MyoG*), mitochondrial biogenesis (*PGC1α*), or fiber type (*Myh7*, *Myh2*, and *Myh4*, which are expressed in type 1 slow fibers, type 2A fibers, and fast type 2B fibers, respectively) markers in quadriceps muscles at 8 and 24 weeks.

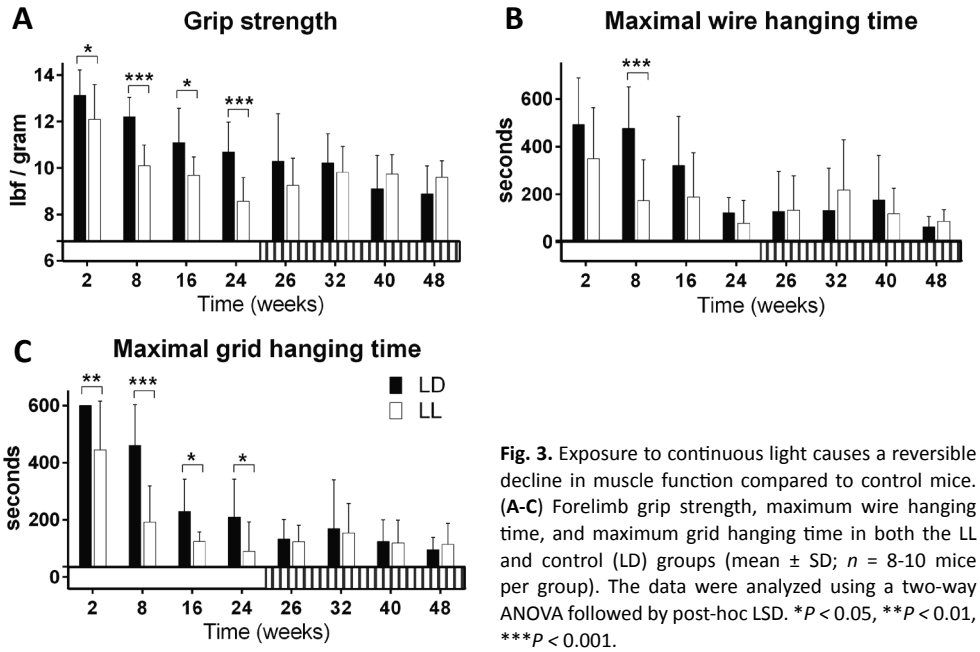
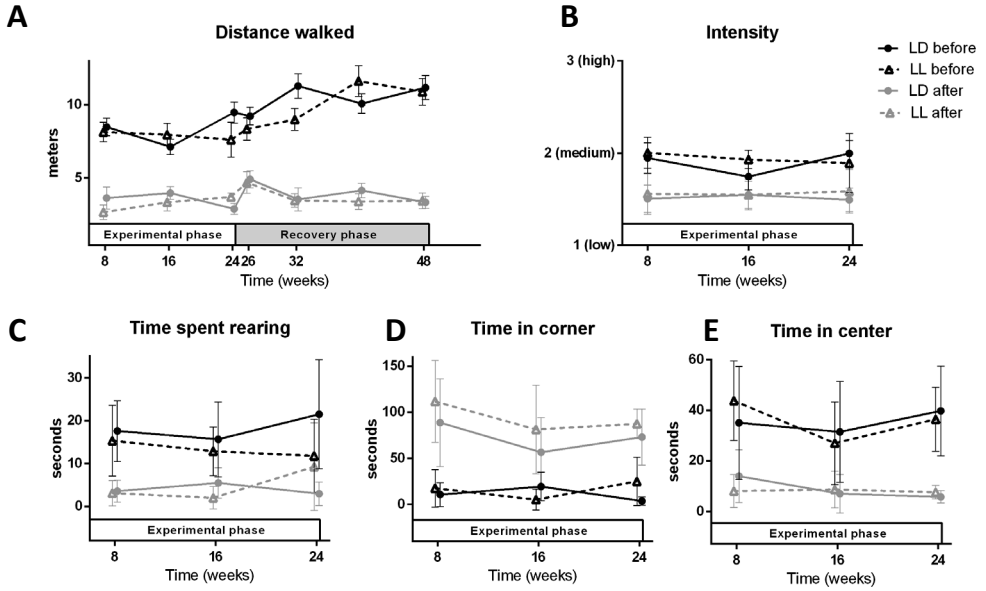


Fig. 3. Exposure to continuous light causes a reversible decline in muscle function compared to control mice. (**A-C**) Forelimb grip strength, maximum wire hanging time, and maximum grid hanging time in both the LL and control (LD) groups (mean ± SD; *n* = 8-10 mice per group). The data were analyzed using a two-way ANOVA followed by post-hoc LSD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Exposure to continuous light induces features characteristic of early osteoporosis

After 8 weeks in the experimental phase, both groups of mice had normal bone maturation and reached their peak relative bone volume density (BV/TV) at 20 weeks of age (**Fig. 4A, Suppl. Fig. 3A-I**). After 8 weeks of LL exposure, the metaphysis and diaphysis of the femurs had a thicker cortex compared to controls (**Suppl. Fig. 3G-I**). Over the subsequent weeks, the volume, thickness, number, and separation of the trabeculae, the structural model index (SMI), and BV/TV began to differ between the two groups (**Suppl. Fig. 3A-E**). At the end of the 24-week experimental phase, the trabeculae of the mice in the LL group were 34%

smaller in volume and 10% thinner (**Fig. 4B-C, Suppl. Fig. 3A-B**). The mice in the LL group also had 28% fewer trabeculae, which were separated more (by 16%). The trabeculae in this group were also more rod-like in shape compared to the control group. Together, these findings are characteristic of the early stages of osteoporosis.



Suppl. Fig. 2: Continuous exposure to light does not affect fatigue or anxiety-related behavior measured before and after functional muscle tests. Distance walked, intensity of behavioral activity, time spent rearing, time spent in the corner, and time spent in the center of the cage were measured directly prior to (black symbols) and after (gray symbols) functional muscle tests were performed (mean ± SEM; *n* = 8-10 mice per group). All parameters were analyzed in 1-second bins from 3-minute video recordings of each mouse; the observer was blinded with respect to the group and time point. Behavioral intensity was scored subjectively as low (1), medium (2), or high (3). Generalized linear models revealed no significant differences between the groups.

In the LL group, cortical bone thickness in the metaphysis was increased by 5%; however, LL exposure had no effect on cortical volume in the metaphysis or any cortical parameters in the diaphysis (**Fig. 4B-C, Suppl. Fig. 3F-I**). Remarkably, bone structure no longer differed between LL-exposed mice and controls after returning the mice to a standard LD cycle (**Suppl. Fig. 3A-I**). Similar results were obtained when we repeated our analyses after controlling for body weight and behavioral activity levels. Lastly, consistent with age-related osteoporosis, the serum levels of calcium, phosphate, and creatinine did not differ significantly between the LL and control groups (**Suppl. Fig. 3J-L**).

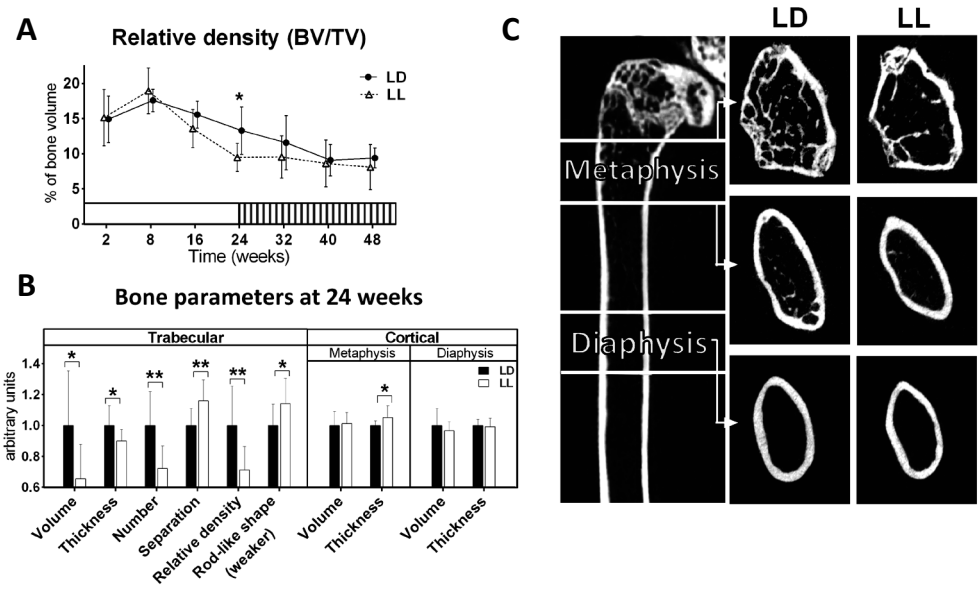
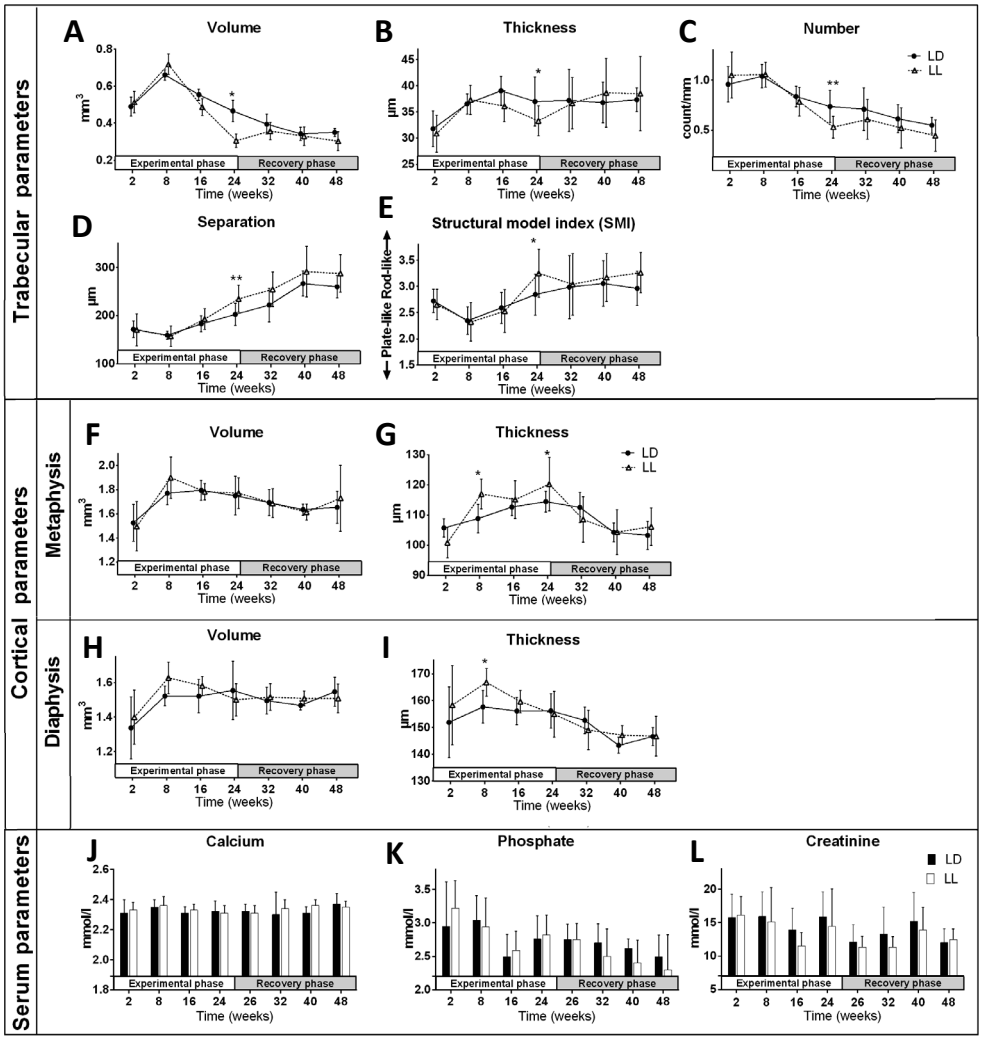


Fig. 4. Continuous exposure to light induces clinical features that are characteristic of early osteoporosis. (**A**) Relative trabecular density (BV/TV, measured as the ratio of bone volume to total volume) was measured in the LL and control groups at the indicated time points (mean ± SD; *n* = 8-9 mice per group). (**B**) Trabecular and cortical bone parameters measured at the end of the experimental phase (i.e., after 24 weeks) in LL and control mice (mean ± SD; *n* = 8-9 per group). All six trabecular bone parameters were significantly worse in the LL group. (**C**) Examples of cross-sectional microCT views of the proximal and distal metaphysis and diaphysis in LL and control mice at 24 weeks. The data were analyzed using a two-way ANOVA followed by post-hoc LSD. **P* < 0.05, ***P* < 0.01.

Exposure to continuous light alters the immune system

The total white blood cell (WBC) count was relatively unchanged throughout the experimental phase and did not differ significantly between groups (**Fig. 5A**). In contrast, both hemoglobin levels and hematocrit values were lower in the LL mice, particularly after 8 weeks of LL exposure (**Fig. 5B-C**). Also at 8 weeks, the relative number of neutrophils was nearly twice as high in the LL group, and the LL mice had fewer lymphocytes (**Fig. 5D-E**). The fraction of monocytes was higher in the mice in the LL group, particularly at 16 weeks (**Fig. 5F**). In the recovery phase, the level of monocytes returned to control levels; however, the hemoglobin and hematocrit values did not return fully to control levels. With respect to the absolute number of leucocyte subtypes, we also measured significantly more neutrophils and significantly fewer lymphocytes in the LL group at 8 weeks; in contrast, absolute number of monocytes did not differ significantly between the two groups.

After 2 weeks into the experimental phase, the mice in the LL group had lower plasma levels of IL-1β and IL-10, and they had higher plasma levels of TNF-α compared to controls (**Fig. 5G**).



Suppl. Fig. 3: Continuous exposure to light induces changes in several bone-related characteristics. (**A-E**) Trabecular bone parameters, (**F-I**) cortical bone parameters, and (**J-L**) serum levels were measured in both LL and control (LD) mice at the indicated time points (mean ± SEM; *n* = 8-9 mice per group). The data were analyzed by two-way ANOVA followed by post-hoc LSD. **P* < 0.05, ***P* < 0.01.

Total cytokine secretion measured as the area under the curve (AUC) also differed for IL-1β and TNF-α (**Fig. 5G, Table 1**). After 8 weeks, LPS-induced secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α was higher in the LL group, whereas secretion of the anti-inflammatory cytokine IL-10 was lower in the LL group. At 24 weeks, LPS-induced cytokine secretion no longer differed between the two groups. Taken together, these data suggest that LL exposure induces a transient pro-inflammatory state.

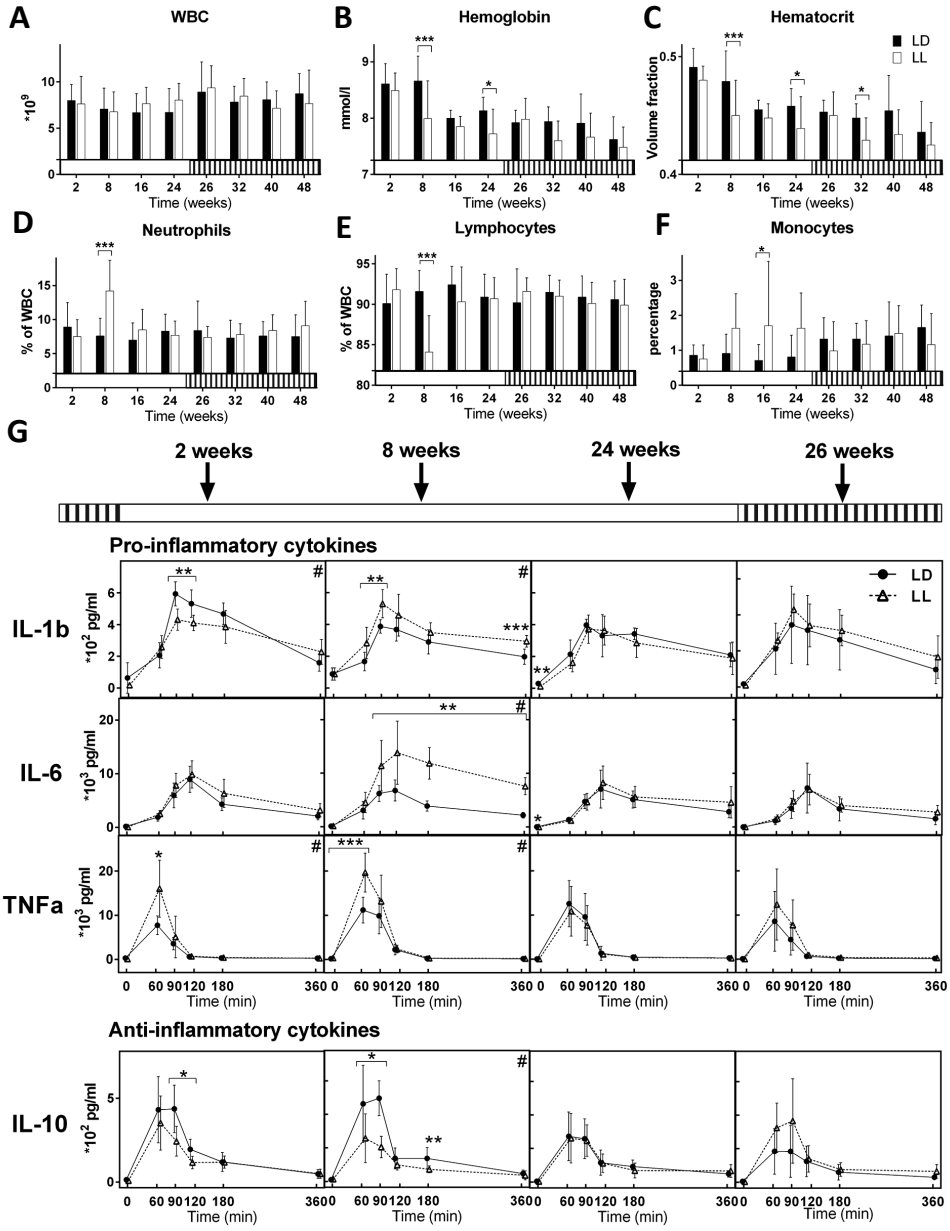


Fig. 5. Continuous exposure to light reversibly alters the homeostatic and responsive states of the immune system. (**A-F**) The indicated values were measured in LL and control mice at the indicated times. WBC, white blood cell count (mean ± SD; *n* = 8-10 mice per group). (**G**) Plasma levels of IL-1β, IL-6, TNF-α, and IL-10 were measured prior to (time 0) and 60, 90, 120, 180, and 360 minutes after an injection of low-dose LPS at the indicated experimental time points (mean ± SD; *n* = 5-12 per group). The data were analyzed using a two-way (**A-F**) or repeated-measures (**G**) ANOVA, followed by post-hoc LSD. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. # in the top-right corner in panel G indicates that the area under the curve differed significantly between the two groups.

Table 1: Altered total cytokine production in response to LPS challenge in continuous light.

	2 wks		8 wks		24 wks		26 wks	
	LD	LL	LD	LL	LD	LL	LD	LL
Pro-inflammatory cytokines								
IL-1β ($x10^4$)	6.7 ± 0.9	5.0 ± 1.0**	4.7 ± 1.0	6.2 ± 1.6**	4.8 ± 0.7	4.4 ± 1.0	3.4 ± 2.6	5.0 ± 1.1
IL-6 ($x10^5$)	8.0 ± 2.3	8.5 ± 2.8	7.5 ± 1.8	13.8 ± 5.6**	6.4 ± 2.2	7.3 ± 2.3	4.7 ± 3.6	6.5 ± 2.3
TNF-α ($x10^5$)	4.7 ± 1.7	9.2 ± 4.1*	9.1 ± 3.5	14.1 ± 3.7**	9.5 ± 4.4	7.9 ± 4.1.0	4.5 ± 4.4	8.5 ± 5.6
Anti-inflammatory cytokine								
IL-10 ($x10^4$)	4.4 ± 1.4	5.8 ± 9.2	4.6 ± 1.8	2.5 ± 0.7**	2.9 ± 1.2	2.6 ± 1.2.0	1.7 ± 1.5	3.4 ± 1.7

The area under the curve (AUC) of each cytokine is shown in pg/ml/min. Data are represented as mean ± SD (*n* = 5-12 per group). Two-way ANOVA, followed by post-hoc LSD. Difference between groups at $*P < 0.05$, $**P < 0.01$.

Discussion

Exposing *C57BL/6J* mice to continuous light for 24 weeks had significant effects on the *in vivo* activity of neurons in the SCN. In addition, continuous light caused a significant decrease in skeletal muscle function and caused microstructural bone changes characteristic of the early stages of osteoporosis. Finally, mice exposed to continuous light displayed transient changes in the immune system, including a pro-inflammatory state that resolved after prolonged exposure to light. Importantly, nearly all of the physiological changes induced by long-term exposure to continuous light were reversed upon re-exposing the mice to a light-dark cycle. Taken together, these results suggest that removing cyclic environmental cues causes a reversible state of physiological vulnerability.

Exposure to continuous light decreases the strength of circadian rhythms

Retinal photoreceptors receive light and project light-encoded information to the SCN via melanopsin-containing retinal ganglion cells and chemical photoreceptors.[25,26] Continuous exposure to light causes desynchronization of SCN neurons,[22] and in peripheral bodily tissues.[27] The long-term effects of LL—as well as the system's capacity to restore rhythmicity after returning to a standard light-dark cycle—had not been studied previously and are highly relevant, given that circadian rhythms can be disrupted for extended periods, for example in shift workers or elderly persons. We confirm that short-term exposure to LL decreases neuronal amplitude[20] and demonstrate that long-term LL exposure further attenuates rhythmicity by approximately two-thirds throughout the 24-weeks of exposure. The strength of the SCN's rhythm of a given animal corresponded temporally with the strength of that animal's behavioral activity rhythm, suggesting that fluctuations in rhythm strength have a direct impact on overt behavioral rhythms.

Continuous light exposure disrupts skeletal muscle function

All mice had a decline in muscle function over time; this progressive decline in muscle function is likely due to the effects of aging.[28] The mice in the LL group had less grip strength than age-matched control mice, indicating that LL reduces muscle strength in addition to age. The mice in the LL group also had reduced physical endurance, reflected by decreased performance on the hanging tests, which require the mouse to maintain sustained force against gravity. Mice use all four limbs for hanging on a grid; in contrast, hanging on a wire is more complex and requires the coordination of several muscle groups, as well as dexterity and axial muscle strength. Even after two weeks in continuous light, the mice in the LL group showed a significant deficit, and this deficit remained throughout the experimental phase. Importantly, this decreased muscle function was independent of body weight or behavioral activity.

A video analysis of mouse behavior prior to and after functional testing revealed that the two groups of mice were fatigued to a similar extent by the testing regime. They displayed similar levels of anxiety-related behavior, suggesting that the LL-induced decline in muscle function was not due to differences in motivation. We currently have no evidence to suggest that the decreased muscle function in the LL group was due to structural differences in muscle fibrosis, muscle damage (e.g., CK levels), macrophage levels in the muscle, muscle fiber type, or differences in mitochondrial function or muscle regeneration. Previous studies have linked circadian rhythm with muscle function by examining mice with mutations in their clock genes; specifically, these mice develop structural muscle changes[1,29,30] and diminished muscle function.[1,31] However, because clock gene mutants may have impaired muscle function due to a direct effect of the clock gene mutation on the cell cycle,[32] these models do not necessarily simulate disruptions in environmental rhythmicity. Our data therefore provide first evidence, that robust external circadian rhythms support muscle function.

Continuous exposure to light induces clinical features reminiscent of early osteoporosis

Short-term (i.e., up to 8 weeks) exposure to continuous light had no effect on bone microstructure.[33] Indeed, the maturation of bone in both animal groups was similar to bone maturation in humans.[33,34] Long-term exposure to LL had a negative effect on the microstructure of cancellous (i.e., trabecular) bone; after 24 weeks, the mice in the LL group had fewer trabeculae. The remaining trabeculae were more rod-like in shape, thinner, less voluminous, and more separated, thereby resulting in decreased BV/TV compared to control mice. These changes in trabecular bone are particularly striking, as C57BL/6 mice have a relatively low BV/TV compared to other mouse strains.[33] The progressive loss of trabecular bone is believed to play the most important role in the decline in bone strength associated with age-related osteoporosis.[35] The increased thickness of cortical bone in mice exposed to continuous light may also be an accelerated effect of aging, as cortical bone mass increases

with age due to periosteal bone formation[35]; alternatively, it may represent a compensatory mechanism designed to maintain overall bone strength. Given its relatively large surface area, trabecular bone undergoes considerable turnover and remodeling; thus, osteoporotic changes are usually observed in trabecular bone first. Therefore, we feel that the changes in trabecular bone in mice exposed to continuous light are characteristic of early osteoporosis.

Of note, the levels of both calcium and phosphate were unaffected in the mice in the LL group, consistent with age-related osteoporosis in humans.[2] Moreover, general kidney function, which was assessed by measuring creatinine levels, was also unaffected by continuous light exposure.

In human studies, female shift workers have an increased risk of bone fractures[12] and decreased bone mineral density.[11] These observational studies cannot be used to determine causality, thus our results indicate for the first time that disrupted environmental rhythms are a causal factor in the decline in bone microstructure.

Circadian disruption and the immune system

Two weeks of LL exposure mildly affected LPS-induced cytokine secretion. Eight weeks of LL exposure induced a higher numbers of neutrophils, pro-inflammatory cells in the innate immune system; thus, LL appears to induce a heightened pro-inflammatory state. Consistent with this notion, the mice in the LL group had higher LPS-induced secretion of the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, coupled with decreased secretion of the anti-inflammatory cytokine IL-10. These differences were no longer observed after 24 weeks of LL exposure, suggesting that the effect of LL on the immune system is transient and/or that compensatory mechanisms may have been activated. Identifying such compensatory mechanisms would provide valuable information.



Previous studies have linked disruptions in environmental rhythms with impaired immune function. For example, shift workers have an increased risk of cancer[9] and metabolic syndrome,[36] both of which are related to immune system dysfunction.[37] Shift workers do not have altered baseline cytokine levels[38] and their immune response to challenges has not been investigated. Previous animal studies have suggested that circadian disruption is a causal factor in altered immune system function. For example, mice subjected to a chronic (i.e., 4-week) jet lag protocol have an enhanced response to LPS challenges[4] and intestinal irritant–induced colitis is more aggressive in mice that are chronically phase-shifted.[39] Moreover, exposing rats to continuous light increases mortality following LPS-induced sepsis.[40] In our experiments, we used a low, non-lethal dose of LPS; therefore, we were able to quantify the effects of an immune stimulus that more closely resembles the inflammatory response in human sepsis.[41]

Recovery of health vs. stability of health upon returning to a normal environmental cycle
After returning to a standard light-dark cycle (i.e., the recovery phase), the mice in the LL group no longer had impaired muscle performance or deficits in trabecular bone microstructure. It is difficult to assess whether restoring the light-dark cycle leads to a bona fide recovery of health, as bone microstructure and muscle function naturally decline with age. Nevertheless, in the recovery phase, many health parameters either stabilized or improved slightly, and none of the parameters measured continued to decline while muscle function and bone microstructure have the potential to decline to much lower levels, as observed in very old mice or models of severe disease.[33,43]

In the SCN, neuronal rhythmicity recovered instantaneously after returning the mice to a standard light-dark cycle. Importantly, this rapid recovery led to a large amplitude rhythm that was properly phased. The trough upon the first exposure to darkness was particularly large, suggesting that this sudden, first absence of light input acts as a "phase-resetting" stimulus for the majority of SCN neurons. Our results are consistent with a previous report in which the SCN's rhythm recovered almost immediately following a short bout of continuous light exposure.[43] Because neuronal activity is the first step in generating the output signal, and because this activity drives the release of both neurotransmitters and humoral signals, restoring the SCN's output signal will have immediate consequences for all peripheral systems that receive either direct or indirect input from the SCN.

Clinical relevance
Exposing animals to continuous light is an important model for intensive care settings and nursing homes, in which lighting can fluctuate so little throughout the 24-hour period that patients usually fail to entrain to these cycles.[44-46] For example, rhythms in behavior, body temperature, corticosteroid levels, heart rate, and melatonin levels are often disrupted—or even abolished—in intensive care patients.[47-50] Ironically, exposure to a robust environmental cycle may be particularly relevant to severely ill patients, as these patients could benefit considerably from a robust immune response. Studies have shown that preterm infants in neonatal intensive care units have improved sleep patterns and gain weight faster when exposed to a robust light-dark cycle.[51-54] In addition, nursing home residents have improved sleep and higher levels of physical activity.[55]

As 18% of elderly adults have decreased muscle strength,[56] 6 - 21% have osteoporosis,[57] and immune system dysregulation can aggravate age-related pathologies,[58] large segments of the elderly population are at increased risk for frailty. A frail state in the elderly could be explained by a decline in their circadian system, since the changes in rhythm amplitude in the SCN in the LL group are reminiscent of rhythm changes that occur in the clock of aged individuals.[59] Therefore, the LL-induced decline in mice may represent the contribution of an "aged" clock to the age-related decline in health.

Conclusions

Here, we provide insight into the long-term effects of disrupted environmental rhythmicity on several major health parameters, and we provide evidence that the majority of these effects are reversible. We conclude that complex temporal relationships involved in daily fluctuations in muscle function, bone microstructure and immune function are disrupted by exposure to continuous light, and this disruption underlies the observed changes in health. These results create new opportunities for prevention and treatment programs, particularly for frail individuals, such as intensive care patients, nursing home residents and the elderly. Our results are also highly relevant to large segments of the population, as three-quarters of the world's population is routinely exposed to artificial light during the night.[6] We propose that long-term prospective studies should be performed to examine the health effects of increasing diurnal light levels in such settings. For example, in addition to increasing light levels during the day,[60] light exposure during the night can be reduced easily without compromising patient safety.[61] The long-term effects of a robust light-dark cycle on muscle function, bone microstructure, and the immune system are currently unknown in humans. Our study provides compelling evidence that the detrimental effects of chronic continuous light exposure warrant further investigation.

Methods

Study design

Adult male *C57BL/6JOlaHSD* mice (Harlan, Horst, the Netherlands) were group-housed in Macrolon cages (35×36 cm) in sound-tempered chambers; food and water were available *ad libitum*. Mice were housed under constant ambient temperature (22°C). At the age of 12 weeks, the mice were randomly assigned to various groups. The mice in the "LL group" were exposed continuously to a light source (Naturalite light bulb; 76 $\mu W/cm^2$ = 105 lux) for 24 weeks ("experimental phase"); during this phase, mice in the "LD (control) group" were exposed to a 12-hour light/12-hour dark cycle. After the 24-week experimental phase, the mice were exposed to the standard light-dark cycle for an additional 24 weeks ("recovery phase"). Body weight was measured at least every two weeks.

The mice in the LL and control groups were further divided into three subgroups. One group of mice was used for muscle and bone experiments; these animals were dissected at 2, 8, 16, 24, 26, 32, 40, and 48 weeks relative to the start of the experimental phase. A second group of mice was subjected to a lipopolysaccharide (LPS) challenge at 2, 8, 24, and 26 weeks. In the third group of mice, *in vivo* SCN neuronal activity was recorded longitudinally (n = 9). All experiments were performed at circadian time 0 (CT0), which corresponds to the start of the animal's active phase. A total of 271 mice were used for these experiments.

Behavioral data

Two weeks prior to beginning the experiments, the mice were transferred to individual cages equipped with passive infrared (PIR) motion detectors (Hygrosens Instruments, Löffingen, Germany) placed underneath the lid of the cage. Because the location of the nest can influence total activity levels and rhythm strength, we ensured that the nest was located at the end of the cage furthest from the PIR sensor by rotating the cage when necessary. During the experiments, the nest was in the optimal location for all but three mice. The PIR detectors were connected to a ClockLab data collection system (Actimetrics, Wilmette, IL). Data recorded on the first day of the experiments were excluded from the analysis. The strength and period of the behavioral rhythm were determined by F periodogram analysis of PIR recordings in 10-minute bins.[62] Rhythm strength was defined as the difference between the 95% confidence limit and the peak in the periodogram. The total number of counts per period was averaged for the individual mice and then adjusted to a 24-hour period (e.g., a value of 1000 counts during 25 hours was adjusted to 960 counts per 24 hour period).

Muscle function tests

Four days before dissection, muscle function tests were performed at CT0 in accordance with the TREAT-NMD Network protocol (http://www.treat-nmd.eu/resources/research-resources/dmd-sops.[42] Forelimb grip strength was measured using a grip strength device (Columbus Instruments, Columbus, OH) in five trials consisting of three attempts, with a two-minute interval between each trial. The three highest values were then averaged. During the wire hanging test, the mice had three attempts to hang for 10 minutes from a rigid metal wire. For the grid hanging test, the mice were placed on an inverted grid in order to assess maximum grid hanging time; the mice had three attempts to hang for 10 minutes. The mice were allowed to rest for three minutes between each attempt, with 45 minutes between each test. Before the muscle function tests, unfasted glucose levels were measured from a drop of blood obtained via a small tail cut; glucose was measured using glucose strips (Accu-Chek Aviva, Reeuwijk, Netherlands). Blood was also collected in 0.3 ml Microvette CB300 tubes before and after the functional testing; the blood was centrifuged at 4°C for 5 minutes at 13,000 rpm. Plasma creatine kinase (CK) levels were measured using Reflotron CK test strips in a Reflotron Plus machine (Roche Diagnostics Ltd., Burgess Hill, UK) within three hours of collection. The mice were also video-recorded using an AXIS 221 Network Camera for three minutes in a new cage before and directly after the muscle function tests. Both cages contained the scent of other mice to stimulate exploratory behavior. Video analyses were performed by a researcher who was blinded with respect to the experimental conditions and time point. Total walking distance was determined manually using Mousetracker software (SuperEasy GmbH & Co, www.supereasy.net). The intensity of behavioral activity was scored per second as “1” (least intense), “2” (moderately

intense), or "3" (most intense). The time spent in the corner, in the center, and rearing were also measured.

Dissection and tissue processing

Mice were euthanized under deep anesthesia with 50 mg/kg ketamine and 0.5 mg/kg dexdomitor, and blood was collected by retro-orbital bleeding. Blood was collected in Microtainer tubes containing K_2EDTA (Becton Dickinson B.V., Breda, the Netherlands) for whole blood analysis and in uncoated Eppendorf tubes for serum analysis. The quadriceps muscles were dissected, snap-frozen in 2-methylbutane (Sigma-Aldrich, Zwijndrecht, the Netherlands), then stored in liquid nitrogen. Femurs were fixed in 4% paraformaldehyde for 48 hours and then stored in 70% ethanol at 4°C.

Serum bone marker analysis

Serum levels of calcium, phosphate, and creatinine were measured with a Modular P800 chemical analyzer (Roche Diagnostics, Indianapolis, IN), using a colorimetric (calcium and phosphate) or enzymatic (creatinine) method. The analytical variability of each test is <2.5%.

Muscle histology

Sections (8-µm thick) were cut using a Shandon cryotome (Thermo Fisher Scientific, Waltham, MA) and affixed to Superfrost Plus slides (Thermo Fisher Scientific). The remaining muscle tissue was collected in tubes containing MagNa Lyser Green Beads (Roche Diagnostics) and used to isolate RNA for qPCR experiments. The sections were stained with goat anti-Collagen type 1 primary antibody (dilution 1:100; 1310-01 Southern Biotech, Birmingham, AL) followed by donkey anti-goat Alexa594 secondary antibody (dilution 1:1000; Invitrogen Life Technologies Europe B.V., Bleiswijk, the Netherlands); the nuclei were counterstained with DAPI. The sections were examined using a model DM5500 B fluorescence microscope (Leica Microsystems B.V., Son, the Netherlands) at 10x magnification, and images covering the entire muscle were captured using a DC350 FX digital camera. The percentage of fibrotic tissue was quantified using ImageJ software (National Institutes of Health, Bethesda, MD; downloaded from http://rsb.info.nih.gov/ij/).

qPCR

The quadriceps muscle was obtained at 8-week and 24-week time points as described above. Total RNA was isolated from the samples using the phenol-chloroform extraction method (Tripure RNA Isolation Reagent, Roche) and quantified by NanoDrop. First-strand cDNA was synthesized from 1 µg of total RNA using the Superscript First-strand Synthesis Kit (Thermo Fischer Scientific). Real-time PCR assays were performed using specific primer sets (sequences provided upon request) with SYBR Green in a CFX Real-Time PCR system

(Bio-Rad, Veenendaal, the Netherlands). mRNA levels were normalized to the housekeeping gene *Gaphd* and are expressed as arbitrary units.

MicroCT measurements

Femurs stored in 70% ethanol were scanned using a Skyscan 1076 X-ray microtomograph (Skyscan, Aartselaar, Belgium). Scan parameters included a 40-kV, 250-µA X-ray source with a 1-mm Al filter (to reduce beam hardening artifacts) and a step size of 0.8° covering a trajectory of 180°. Images were reconstructed at 9.12-µm isotropic voxel size using NRecon software (V1.6.2.0, Skyscan) with ring artifact correction set to 5 and beam hardening correction set to 20%. Femurs were reoriented with the mid-diaphysis parallel to the *z*-axis, and 15-mm long regions were selected in the diaphysis and distal metaphysis (0.3 mm under the epiphysis). Reconstructed grayscale images were segmented using an automated algorithm with local thresholds[63] in order to distinguish calcified tissue from non-calcified tissue. The trabeculae and cortex were identified using the software program PrStackBot-New (Orthopedic Research Laboratory, Erasmus Medical Center, Rotterdam, the Netherlands). Bone morphometric parameters were determined in the distal metaphysis and the diaphysis using 3D-Calculator (Skyscan).

Whole blood analysis

Whole blood was analyzed within six hours of collection using an automated Sysmex XE-2100 blood cell counter (Sysmex Cooperation, Kobe, Japan). Total and differential white blood cell (WBC) counts were determined using flow cytometry with forward-scatter, side-scatter, and lateral fluorescent light.[64] Hemoglobin and hematocrit values were measured using the sodium lauryl sulfate-hemoglobin and RBC pulse-height detection methods, respectively. The results were confirmed by performing a light-microscopy examination of hematoxylin and eosin–stained blood smears from ten mice.

LPS challenge and cytokine analysis

At CT0, mice were injected intravenously with 50 µg/kg LPS (*E. Coli* O111:B4, purified by ion-exchange chromatography, catalog number L3024, Sigma, St. Louis, MO). Tail blood was collected in 0.3 ml Microvette CB300 tubes directly prior to LPS injection and 60, 90, 120, 180, and 360 minutes after injection; after collection, the blood samples were centrifuged at 1,000 rpm for 15 minutes. The supernatant was collected and centrifuged at 10,000 rpm for 10 minutes; this supernatant was stored at -80°C. Plasma cytokine levels were measured using the Bio-Plex Protein Array System and a mouse cytokine panel (Bio-Rad). IL-1β, IL-6, IL-10, and TNF-α concentrations were measured using the Bio-Plex Manager software program. The AUC was calculated for each cytokine at each time point.

In vivo SCN electrophysiology

At an age of 12 to 15 weeks , a separate group of mice was anesthetized with 100 mg/kg ketamine, 20 mg/kg xylazine, and 1 mg/kg atropine, and a tripolar stainless steel electrode (PlasticsOne, Roanoke, VA) was implanted using a stereotaxic setup (Stoelting Europe, Dublin, Ireland). Two polyimide-insulated electrodes were aimed at the SCN (0.61 mm lateral of Bregma, 5.38 mm below the dura, at a 5° angle) for differential neuronal recordings, and a reference electrode was placed in the cortex. Following a one-week recovery period, the electrodes were connected to a custom-made recording system via a flexible cable with a counterbalanced swivel system that enabled the animals to move freely. After the signal was amplified and filtered (0.5-5 kHz), action potentials were detected with window discriminators and stored in 10-sec epochs (Circa V1.9, custom-made software). Multiunit activity (MUA) was recorded for 3 days in a standard LD cycle and for the first 7 days in LL. Mice were then recorded at 8 and 24 weeks in LL, and upon returning to a regular LD cycle. The mice in the control group were not subjected to continuous light. Behavioral activity was recorded simultaneously using a PIR sensor. Mice were disconnected from the recording system to minimize traction on the electrode. Rhythm strength of behavioral activity was measured using F periodogram analysis. The MUA signal was smoothed using a penalized least-squared algorithm.[65] Amplitude (peak height minus the average height of the adjacent troughs) was measured from the smoothed signal. After recording, the animals were sacrificed, and a small electric current was passed through the electrode to deposit iron at the site of the electrode tip. The brains were collected and fixed in 4% formaldehyde containing ferrocyanide to stain the iron deposited from the electrode tip. The brains were stained with cresyl violet, and placement of the electrode tip in the SCN was confirmed histologically.

Statistics

All statistical analyses were performed using SPSS 20.0 for windows (IBM Corp., Armonk, NY) or the R 3.0.1 statistics package (Foundation for Statistical Computing, Vienna, Austria) using the VGAM package.[66,67] Normality of data was tested using the Shapiro-Wilk test and Q-Q plots. Two-way ANOVAs were performed and, in case of significance ($P < 0.05$), the Fisher's Least Significant Difference (LSD) procedure was used to compare the LL and control groups at each time point. Analyses were performed separately for the exposure and recovery phases of the experiment. Repeated-measures ANOVA with post-hoc LSD were performed to analyze the longitudinal data. Because the wire hanging and grid hanging trials had a cut-off value of 600 seconds, the effect of LL was examined using Tobit regression with accounting for censored effects. Dummy variables were used to compare the LL and control groups at each time point. In mice that were exposed to LL for 24 weeks and then returned to a standard 12-hour light/12-hour dark cycle, a five-day moving average was

calculated for period and rhythm strength. Pearson's correlation coefficient was calculated to determine the correlation between the rhythm strength of behavior and MUA measured in each mouse. Generalized linear mixed models using a compound symmetry covariance structure were constructed for analyzing the video data. Optimal distributions and link functions were determined using the Akaike Information Criterion.

Study approval
All experiments were approved by the Leiden University Medical Center's Ethics Committee for Animal Experimentation.

Author Contributions

E.A.L., J.H.M., J.S., A.A.R., H.H.S., B.G., and C.W.G.M.L. designed the experiments. E.A.L., M.v.P., S.R.d.K., R.P.M.S., J.G., S.L.V., M.v.d.V., and K.E.d.R. performed the experiments. E.A.L. and J.H.M. wrote the paper, and C.P.C., A.A.R., M.v.P., S.R.d.K., S.L.V., H.H.S., B.G., J.S., K.E.d.R., and C.W.G.M.L. provided comments on the final manuscript.

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CHAPTER 9

Evening chronotype is associated with changes in eating behavior, more sleep apnea, and increased stress hormones in short sleeping obese individuals

Eliane A. Lucassen,[1,2] Xiongce Zhao,[3] Kristina I. Rother,[4] Megan S. Mattingly,[5] Amber B. Courville,[1] Lilian de Jonge,[5] Gyorgy Csako,[6] and Giovanni Cizza,[5] for the Sleep Extension Study Group

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1. Clinical Center, National Institutes of Health, Bethesda, United States.
2. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
3. Intramural Research Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
4. Pediatric Diabetes and Metabolism, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
5. Section on Neuroendocrinology of Obesity, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
6. Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, United States.

Abstract

Background: Short sleep duration and decreased sleep quality are emerging risk factors for obesity and its associated morbidities. Chronotype, an attribute that reflects individual preferences in the timing of sleep and other behaviors, is a continuum from morningness to eveningness. The importance of chronotype in relation to obesity is mostly unknown. Evening types tend to have unhealthy eating habits and suffer from psychological problems more frequently than Morning types, thus we hypothesized that eveningness may affect health parameters in a cohort of obese individuals reporting sleeping less than 6.5 hrs per night.

Methodology and Principal Findings: Baseline data from obese (BMI: 38.5 ± 6.4 kg/m^2) and short sleeping (5.8 ± 0.8 hrs/night by actigraphy) participants (*n* = 119) of the Sleep Extension Study were analyzed (www.ClinicalTrials.gov, identifier NCT00261898). Assessments included the Horne and Ostberg Morningness-Eveningness questionnaire, a three-day dietary intake diary, a 14-day sleep diary, 14 days of actigraphy, and measurements of sleep apnea. Twenty-four hrs urinary free cortisol, 24 hrs urinary norepinephrine and epinephrine levels, morning plasma ACTH and serum cortisol, fasting glucose and insulin, and lipid parameters were determined. Eveningness was associated with eating later in the day on both working and non-working days. Progression towards eveningness was associated with an increase in BMI, resting heart rate, food portion size, and a decrease in the number of eating occasions and HDL-cholesterol. Evening types had overtly higher 24 hrs urinary epinephrine and morning plasma ACTH levels, and higher morning resting heart rate than Morning types. In addition, Evening types more often had sleep apnea, independent of BMI or neck circumference.

Conclusions: Eveningness was associated with eating later and a tendency towards fewer and larger meals and lower HDL cholesterol levels. In addition, Evening types had more sleep apnea and higher stress hormones. Thus, eveningness in obese, chronically sleep-deprived individuals compounds the cardiovascular risk associated with obesity.

Introduction

The prevalence of obesity, 36% among adults in the US, has reached epidemic proportions.[1] The actual reasons for this increase, in addition to eating more and moving less, are not clear.[2] Therefore, identification of causative factors contributing to the obesity epidemic is of great public health importance. In temporal association with the obesity epidemic, an increasing percentage of the US population, from 20% in 1985 to 25% in 2004, has been reporting sleeping less than six hrs per night.[3] Because of the co-occurrence of these two phenomena, we and others hypothesized that chronic sleep deprivation and weight gain may be causally related to each other.[3] Recently, an epidemiological study of approximately 65,000 individuals conducted in Europe demonstrated that, in addition to sleep duration, individual predisposition in sleep timing influences body weight.[4]

Individual preference for sleep timing and timing of other behaviors is a stable trait, referred to as one's chronotype. A large twin study demonstrated that approximately 50% of the chronotype features are heritable.[5] In addition to a genetic component, several demographic and developmental factors such as age, ethnicity, and gender influence the expression of chronotype: eveningness preference reaches a peak around age20 and then progressively moves towards morningness, with men slightly more evening oriented than women.[5,6] In 1976, Horne and Ostberg developed a questionnaire to classify individuals based on their preferences for sleep timing.[7]

In "modern" regimented societies sleep timing, especially during working days, is influenced by social norms, a phenomenon known as "social jet lag" and we are often forced to be awake against our preferred times. Evening types tend to get less and more shallow, non-refreshing sleep during working days.[6,8] In addition, Evening types experience anxiety and negative mood, tend to have lower self-esteem, perform worse in school and may be more susceptible to stress.[9,10] In terms of feeding behavior, eveningness has been associated with lower dietary restraint, less healthful dietary habits, and a tendency for a higher body mass index (BMI).[11,12]

Circadian rhythms are oscillations with a period of approximately 24 hrs that are generated in the suprachiasmatic nucleus of the hypothalamus. These endogenous rhythms orchestrate most physiological functions and are synchronized to the environment mainly by sunlight, physical activity, feeding and sleep.[13] The constitutive features of circadian rhythms are best revealed in conditions in which environmental factors are kept invariable. Morning and Evening types at their extreme may be shifted by about two to three hrs in circadian oscillations of many bodily functions, including body temperature, melatonin, cortisol, and other hormones secretion.[14]

In this study, we examined the relationship of chronotype with sleep, food intake, endocrine and metabolic parameters in a cohort of obese subjects who reported sleeping less than 6.5 hrs per night.

Methods

Study design and participants

This analysis is part of the Sleep Extension Study, a prospective, randomized, controlled study of obese (BMI 30–55 kg/m^2) men and premenopausal women, 18 to 50 yrs old, reporting sleeping less than 6.5 hrs per night. Details of this study have been previously reported.[15] A total of 126 participants were randomized in a 2:1 ratio to the Intervention or the Comparison Group, and the Intervention Group was coached to lengthen sleep and improve sleep hygiene with life-style modifications. Data in this manuscript were obtained at the Screening and the Randomization Visits, thus prior to any behavioral intervention.

A 24 hrs urine collection was started during the first afternoon of the Overnight Randomization Visit. At 8:00 the following day, after an overnight fast, blood samples, vital signs, and anthropometric measures were obtained. Participants completed the Pittsburgh Sleep Quality Index, Epworth Sleepiness Scale, and the Horne and Ostberg questionnaire.[7,16,17] Sleep apnea recordings were performed for one night .[18,19] Participants were instructed to keep a sleep diary and to wear a wrist actigraphy monitor for the following 14 days. Out of 126 randomized participants, 119 completed the Horne and Ostberg questionnaire and of these 113 recorded dietary intake for three days.

Ethics statement

The study was conducted at the NIH Clinical Center in Bethesda, MD, USA, after approval by the Institutional Review Board of the NIDDK, and is listed in ClinicalTrials.gov (identifier: NCT00261898). Each participant signed a written informed consent form.

Assessments

(A) Chronotype questionnaire.

The Horne and Ostberg questionnaire inquires about preferred sleep time and daily performance (score range: 16–86).[7] It contains 19 questions, such as: “at what time would you like to get up?”, “at what time do you feel tired?”, and “what would be the best time to perform hard physical work?”. Based on their scores, individuals were categorized as being either Morning (score: 50–86) or Evening types (score: 16–49). Chronotype was also analyzed as a continuous variable.[20]

(B) Sleep measurements.

Sleep duration was assessed by a wrist actigraphy monitors (Actiwatch-64, Mini Mitter/ Respironics/ Philips, Bend, OR) that participants (n = 115) wore constantly for recording gross locomotor activity in one-minute epochs.[21] Eighty-nine participants provided information on whether the days were working or non-working. Bedtime and risetime were the first and last 10-min period with less than one “active” epoch, respectively. Bedtime

and wake-up time were estimated by the investigator according to sleep diaries. When no diaries were available (*n* = 16/119), best judgment was used. Actiwatch data were analyzed by Actiware-Sleep version 3.4 software. Sleep efficiency was defined as the percentage of time sleeping out of total time in bed. Mid sleep time was the clock time of the center of the sleep duration period, which was calculated by adding half the sleep duration to the bedtime. The Pittsburgh Sleep Quality Index and the Epworth Sleepiness Scale are questionnaires that assess sleep quality and daytime sleepiness (score ranges: 0–21 and 0–24 with abnormal thresholds of 5 and 10, respectively).[16,17] A portable screening device (Apnea Risk Evaluation System, Advanced Brain Monitoring Inc, Carlsbad, CA) provided an estimate of the respiratory disturbance index (RDI): the number of apneas and hypopneas per hr of sleep.[18,19] An RDI greater than 5 episodes per hr is considered abnormal. The information on RDI score was available in 100 participants.

(C) Anthropometric parameters.
Height was measured to the nearest centimeter using a wall-mounted stadiometer (SECA 242, SECA North America East, Hanover, MD) and weight was measured using a stand-on-scale in a hospital gown to the nearest 1/10th of a kg (SR555 SR Scales, SR Instruments, INC, Tonawanda, NY). Neck and waist circumference measurements were done using a non-stretch measuring tape in triplicate to the nearest mm. Neck circumference was measured at the minimal circumference with the head in the Frankfort Horizontal Plane and waist circumference was measured at the uppermost border of the iliac crest.

(D) Clinical laboratory analysis.
Glucose, total cholesterol, and triglycerides were determined with enzymatic methods highdensity lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C) with direct homogeneous methods in fasting serum specimens on an automated analyzer (Dimension Vista 1500, Siemens Health Diagnostics, Deerfield, IL). Plasma ACTH, serum cortisol, and insulin levels were assessed with chemiluminescence immunoassays (Immulite 2500, Siemens). Urinary free cortisol and catecholamines were measured in 24 hrs collections with liquid chromatography-tandem mass spectrometry, and high-performance liquid chromatography, respectively.



(E) Dietary assessment.
Participants recorded food intake for three consecutive days, preferably two weekdays and one weekend day. At the Screening Visit, they received written and verbal instructions on recording food intake and were instructed to begin record everything they ate or drank beginning when they woke up on the first day until they woke up then next morning. At the Randomization Visit, dietitians and health technicians reviewed the food records with the

participants utilizing three dimensional food models. Information on timing and location of meals was also obtained. The data were analyzed using the Nutrition Data System for Research software (versions 2007–2010, Nutrition Coordinating Center (NCC), University of Minnesota, Minneapolis, MN).[22] Participants also indicated whether days were school- or working days, which we classified as "working days", or holidays or travel days, which we classified as "nonworking days". An eating occasion was defined as containing at least 20 kcal and being at least 30 min apart from another eating occasion.

(F) Statistical analysis.

Normality of variables was examined by Q-Q normality plots. Mean and standard deviation (SD) were calculated for variables with a normal distribution; median and interquartile range were used for variables with a skewed distribution (RDI, plasma ACTH, serum cortisol, HDL-C, LDLC, triglycerides, insulin, 24 h-urinary norepinephrine, 24 hurinary epinephrine, urinary free cortisol, and food intake after 20:00). Logarithmic transformation was applied for skewed variables for further analysis. Morning types were compared to Evening types by independent *t*-tests for continuous variables, and Fisher exac*t*-test for categorical variables. Paired *t*-test was used to compare sleep and food intake parameters on working *vs.* nonworking days.

Relationships between chronotype score and outcome variables were assessed by linear regression, corrected for gender, age, and anthropometric measures, as appropriates. To assess possible predictors of chronotype score, multivariate forward stepwise analyses ($P < 0.05$ to enter, $P \geq 0.10$ to remove) were generated by including each of the variables that correlated with chronotype score in the univariate analysis at $P < 0.10$ (bed- and meal times were not included because they are surrogates for chronotype score). This multivariate model was corrected for age and gender. We tested for the presence of interactions among chronotype score, gender and age. Since we found no interactions, we did not include their product in the model. Analyses were performed using SPSS (version 19, IBM SPSS North America, Chicago, IL).

Results

Demographic and anthropometric characteristics according to chronotype

Approximately two-thirds of participants were Morning types and they were about three yrs older than Evening types (**Table 1**). There were no gender or racial differences between chronotypes. Morning and Evening chronotypes also had similar anthropometrics but the resting heart rate was significantly lower in Morning types. The chronotype score for the whole sample was 53.7 ± 9.9, mean ± SD; $n = 119$ (men: 52.9 ± 9.7; $n = 27$; women: 54.0 ± 10.0; $n = 92$; $P = 0.630$).

Table 1. Demographic and anthropometric characteristics of study participants.

	Morning chronotype (score 50 - 86) *n* = 80	**Evening chronotype (score 16 – 49) *n* = 39**	***p* value**
Chronotype score (range 16 – 86)	59.1 ± 6.8	42.7 ± 5.0	**<0.001**
Age (yrs)	41.7 ± 5.9	38.6 ± 7.8	**0.019**
Female	76%	80%	0.692
African-American/White/Other races (*n*)	54/38/9	72/28/0	0.054* 0.219**
BMI (kg/m^2)	38.2 ± 6.3	39.1 ± 6.6	0.470
Waist circumference (cm)	113.0 ± 13.6	114.7 ± 11.5	0.510
Neck circumference (cm)	38.8 ± 3.8	39.6 ± 3.8	0.340
Morning resting heart rate (beats/min)	68.4 ± 10.1	74.0 ± 10.1	**0.007**

Values are means ± SD, #median and interquartile range for skewed variables, or %. Skewed variables were log-transformed prior to analysis. *p* values for *t*-tests or *Fisher exac*t*-test are depicted and significant *p* values (<0.05) are bolded. *Fisher exac*t*-test including all races; **Fisher exac*t*-test excluding "Other races".

Sleep characteristics

As expected for short sleepers, 29% of the participants experienced excessive daytime sleepiness and 84% experienced abnormal sleep quality, as assessed by the Epworth Sleepiness Scale and the Pittsburgh Sleep Quality Index, respectively (**Table 2**). There were, however, no chronotype differences in sleep quality and sleepiness.

By self-reported diaries, Morning types reported sleeping significantly less than Evening types on working days, a difference that disappeared during non-working days (**Table 2**). In contrast, sleep duration and sleep efficiency by actigraphy were similar between types, both on working and non-working days. As expected, both types slept longer during non-working *vs.* working days, according to either sleep diary or actigraphy measures. Sleep apnea was about two times less prevalent in Morning types than in Evening types.

Fig. 1 depicts chronotype sleep timing by actigraphy. Both on working and non-working days, Morning types went to bed earlier than Evening types and rose earlier. On non-working *vs.* working days, Morning types went to bed at the same time but rose 49 min later ($P < 0.001$), whereas Evening types went to bed 29 min later ($P = 0.019$) and rose 53 min later ($P < 0.001$).

Table 2. Sleep characteristics of study participants.

	Morning chronotype (score 50 - 86) *n* = 80	**Evening chronotype (score 16 – 49) *n* = 39**	***p* value**
Pittsburgh Sleep Quality Index score	8.0 ± 2.7	8.6 ± 2.9	0.263
Epworth Sleepiness Scale score	8.4 ± 4.5	7.6 ± 4.5	0.387
Self-reported sleep duration by diary (min)			
Working days	361 ± 56[1]	403 ± 51	**0.002**
Non-working days	420 ± 63[1]	421 ±39	0.947
Sleep duration by actigraphy (min)			
Working days	339 ± 52[1]	346 ± 64	0.576
Non-working days	386 ± 61[1]	379 ± 60	0.625
Sleep efficiency by actigraphy (%)			
Working days	81.5 ± 5.7	81.3 ± 8.2	0.914
Non-working days	81.1 ± 7.5	81.0 ± 7.8	0.964
Sleep apnea measurements			
RDI (events/hrs)[#]	5 (2 – 12)	10 (7 – 16)	**0.018**
RDI > 5 (events/hrs)	47%	81%	**0.001***

Values are means ± SD, [#]median and interquartile range for skewed variables, or %. Skewed variables were log-transformed prior to analysis. *p* values for *t*-tests or *Fisher exac*t*-test are depicted and significant *p* values (<0.05) are bolded. *Fisher exac*t*-test including all races; **Fisher exact-test excluding "Other races". [1]Sleep duration differed on working *vs.* non-working days in Morning (diary and actigraphy: $P < 0.001$) and in Evening chronotypes (diary: $P = 0.053$; actigraphy: $P = 0.005$).

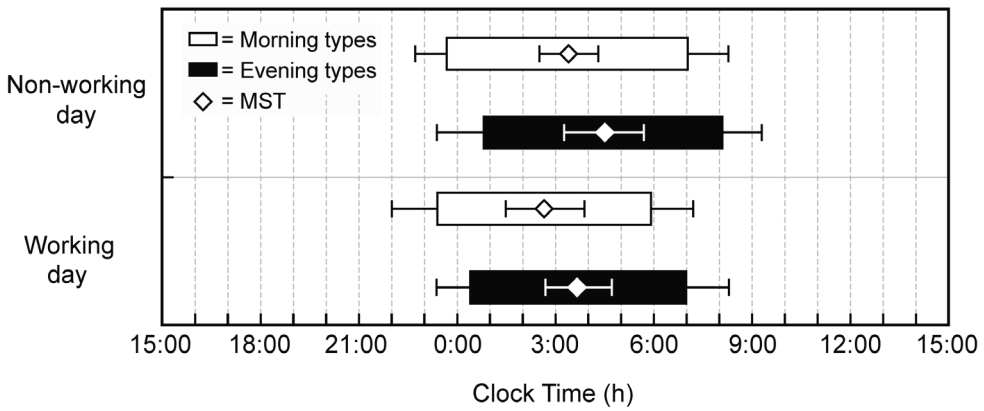


Fig. 1. Sleep characteristics per chronotype. Horizontal bars represent sleep time as measured by actigraphy. Bedtime (mean ± SD) and risetime (mean ± SD) on all days combined and separately on non-working days and on work days in Morning (grey bars) and Evening types (black bars). The mid-sleep times (MSTs) (mean ± SD) are depicted by open diamonds and compared between chronotypes by *t*-tests.

Food parameters

When examining the three day food recall diary, no differences between chronotypes were observed in total energy intake, portion size, or number of eating occasions, albeit during working days Morning types tended to have smaller portions and to eat more frequently than Evening types (**Table 3**). During working days, Morning types ate breakfast approximately 1 hrs and 20 min earlier than Evening types, and after 20:00 they ate approximately 50% fewer calories in fewer eating occasions. A similar pattern was observed during non-working days. Similarly, as chronotype score moved from morningness towards eveningness, participants had fewer eating occasions, larger portions and consumed more calories after 20:00 (**Fig. 2**). Meals consumed after 20:00 contained less carbohydrate (49 ± 16% *vs.* 53 ± 10%; *P* = 0.021) and protein (12 ± 7% *vs.* 14 ± 4%; *P* = 0.006), and tended to contain more fat (34 ± 14% *vs.*32 ± 7%; *P* = 0.069) than meals consumed before 20:00. There were no differences however between chronotypes in the total amount of fat (*P* = 0.14); carbohydrates (*P* = 0.84); or protein (*P* = 0.89) consumed before and after 20:00.

Table 3. Food intake parameters of study participants divided by chronotype on working *vs.* non-working days.

	Working day			Non-working day		
	Morning type	**Evening type**	***p* value**	**Morning type**	**Evening type**	***p* value**
Total food intake (kcal)	2129 ± 631	2276 ± 815	0.373	2383 ± 928	2378 ± 883	0.922
Portion size (kcal)	461 ± 177	545 ± 219	0.065	599 ± 273	622 ± 380	0.751
Eating occasions per day (*n*)	4.9 ± 1.5	4.4 ± 1.5	0.175	4.2 ± 1.2	4.3 ± 1.6	0.568
First eating occasion (h:min)	7:17 ± 1:31	8:38 ± 1:52	**<0.001**	8:56 ±2:30	9:59 ± 2:32	0.075
Food intake after 20:00 (kcal)	299 ± 354	677 ± 460	**<0.001**	327 ± 354	537 ± 480	**0.028**
Food intake after 20:00 (% of total caloric intake)	14 ± 15	30 ± 18	**<0.001**	14 ± 15	24 ± 20	**0.009**

Values are means ± SD. Intake parameters of chronotypes were compared with *t*-tests on working and non-working days separately. Significant *p* values (< 0.05) are bolded.

Relationship between chronotype and anthropometric and lipid parameters

Moving from morningness towards eveningness scores was associated with an increase in BMI, larger neck circumference, and lower HDL-C levels (**Fig. 3**). The effect size was remarkable. For example, a 10-unit change in chronotype score was associated with a change of 1.2 kg/m^2 in BMI (or 3.8 kg in weight), a 0.6 cm change in neck circumference, and a 2.4 mg/dL change in HDL-C levels. Because gender, as expected, was associated with differences in anthropometric measures and serum lipids, all correlations were corrected for gender.

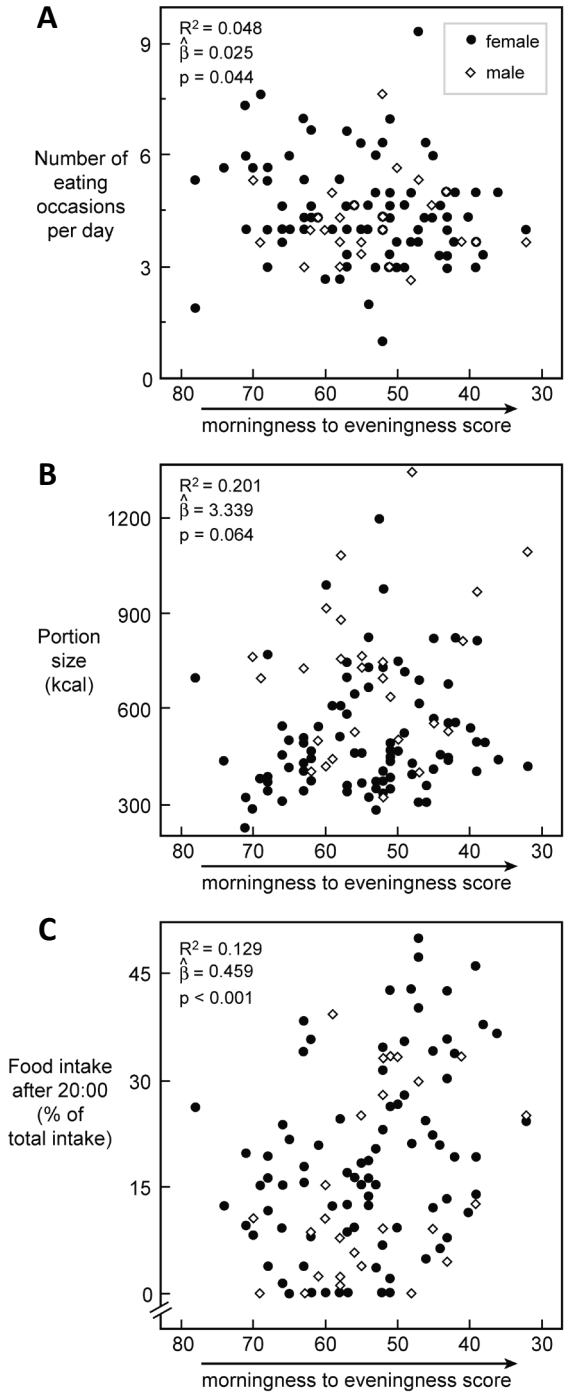


Fig. 2. Pattern and timing of food intake *vs.* chronotype score. Plot of chronotype scores *vs.* the number of eating occasions (**A**), the portion size (**B**), and the amount of caloric intake after 20:00 (**C**) with a trend line, respectively. R^2, slope, and *p* value for the prediction of chronotype score on food intake variables in each plot are calculated from gender-corrected models. Closed circles represent women, open diamonds represent men. *n* = 113.

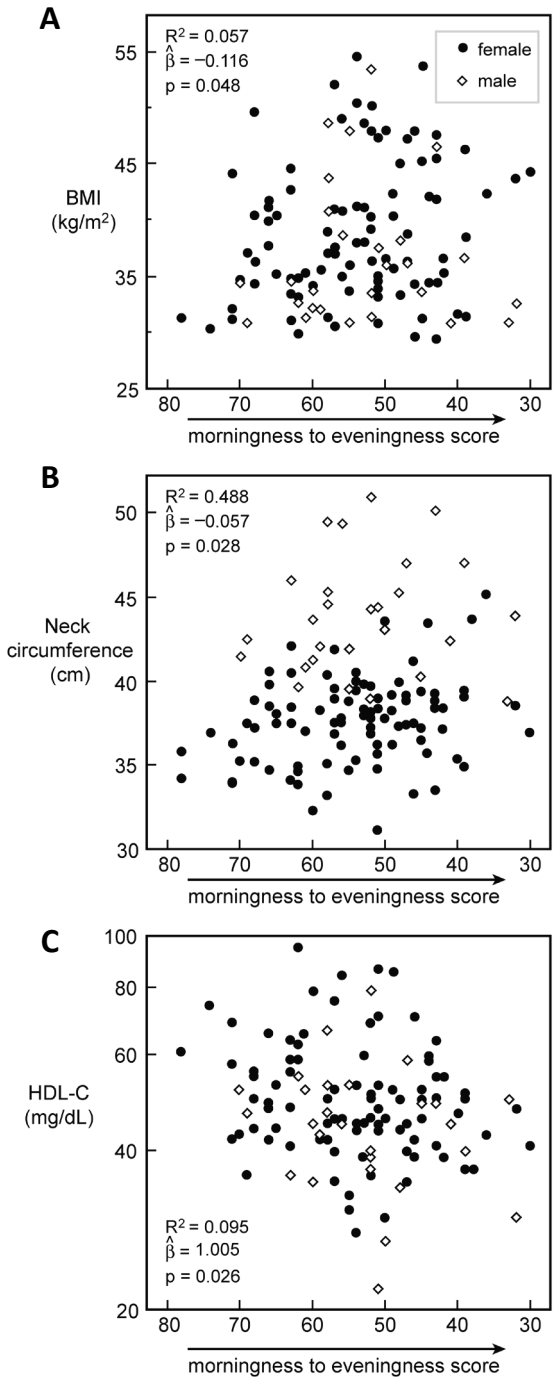


Fig. 3. Anthropometrics and HDL-C *vs.* chronotype score. Plot of Chronotype scores *vs.* BMI (**A**), neck circumference (**B**), and HDL-C (**C**) with a trend line, respectively. n = 119 for all. R^2 slope, and pvalue for the prediction of chronotype score on variables in each plot are calculated from gender-corrected models. Closed circles represent women, open diamonds represent men.

Endocrine and metabolic features

Morning types had higher plasma ACTH and 24 hrs-urinary epinephrine concentrations and tended to have higher levels of 24 hrs-urinary norepinephrine than Evening types (**Table 4**). Morning types had also lower resting heart rates than Evening types. A gender-corrected model elicited similar results. Progression towards eveningness was associated with higher plasma ACTH levels, higher 24 hrs-urinary epinephrine and norepinephrine levels, and a higher heart rate (**Fig. 4**).

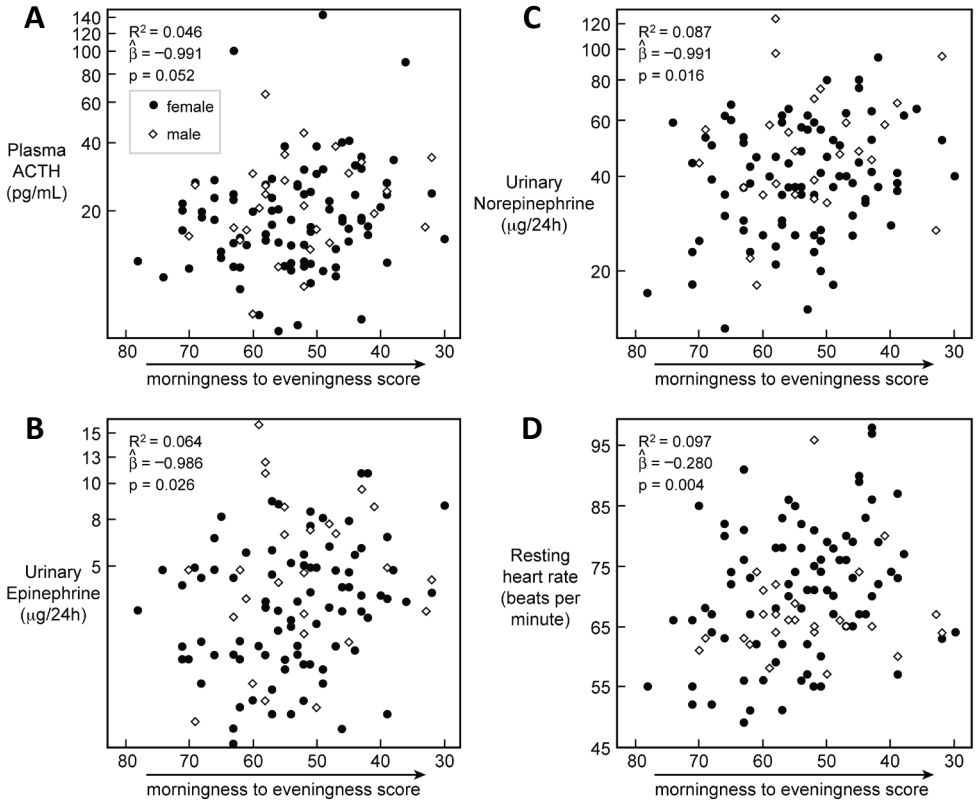


Fig. 4. Stress hormones and heart rate *vs.* chronotype score. Chronotype scores on the horizontal axis are regressed against plasma ACTH (*n* = 118; **A**), 24 hrs urinary epinephrine (*n* = 110; **B**) and norepinephrine (*n* = 114; **C**), and resting heart rate (*n* = 113; **D**). R^2 for the gender-corrected model is reported, and slope and *p* value for the prediction of chronotype score on variables in the model are given. Closed circles represent women, open diamonds represent men.

Table 4. Endocrine and metabolic parameters of study participants.

	Morning chronotype (score 50 - 86) *n* = 80	**Evening chronotype (score 16 – 49) *n* = 39**	***p* value**
Endocrine features			
Morning plasma ACTH (pg/ml)[#,$]	17 (12 – 24)	21 (16 – 32)	**0.019**
Morning serum cortisol (µg/dL)[#,$]	9 (6 – 12)	10 (6 – 14)	0.530
Urinary free cortisol (µg/24hrs)[#]	17 (12 – 24)	19 (10 – 28)	0.885
Urinary norepinephrine (µg/24hrs)[#]	39 (28 – 56)	45 (37 – 61)	0.052
Urinary epinephrine (µg/24hrs)[#]	3 (2 – 5)	4 (3 – 7)	**0.039**
Metabolic features			
Insulin (mU/l)[#,$]	9 (7 – 14)	10 (6 – 16)	0.674
Glucose (mg/dL)[$]	88.6 ± 10.5	90.1 ± 9.6	0.465
Total cholesterol (mg/dL)[$]	178.9 ± 36.7	177.1 ± 35.5	0.806
Triglycerides (mg/dL)[#,$]	91 (62 – 133)	75 (55 – 106)	0.214
HDL-C (mg/dL)[#,$]	48 (42 - 58)	49 (41 – 52)	0.513
LDL-C (mg/dL)[#,$]	106 (91 – 125)	116 (89 – 134)	0.532

Values are means ± SD or [#]median and interquartile range for skewed variables. [$]Measured in fasting morning plasma samples. *p* values for *t*-tests are depicted.

Statistical modeling

RDI was directly related to plasma ACTH (regression coefficient = 0.132; $P = 0.009$) and 24 hrs urinary norepinephrine (coefficient = 0.112; $P = 0.004$). RDI remained inversely related to chronotype score after correction for BMI (coefficient = 20.011, $P = 0.018$) and neck circumference (coefficient =20.009, $P = 0.037$). After correction for BMI, chronotype score remained inversely related to the first eating occasion (coefficient =20.059, $P < 0.001$), caloric intake after 20:00 (coefficient=210.687, $P = 0.001$), percent caloric intake after 20:00 (coefficient =20.445, $P = 0.001$), and HDL-C concentrations (coefficient =20.002, $P = 0.035$), portion size (coefficient = 0.025, $P = 0.052$), and eating occasions (coefficient =23.554, $P = 0.079$).

A gender- and age-corrected multivariate forward stepwise model was fitted with predictors that related to chronotype score at $P < 0.10$ in gender-corrected univariate regressions: sleep duration by diary on working days, RDI, BMI, neck circumference, number of eating occasions, portion size, heart rate, HDL-C, plasma ACTH, 24 hrs-urinary epinephrine and 24 hrs urinary norepinephrine. The following variables did not relate to chronotype score at $P < 0.10$: Pittsburgh Sleep Quality Index, Epworth Sleepiness Scale, sleep duration by diary on non-working days, sleep duration and sleep efficiency by actigraphy on workingand non-working days, total food intake, waist circumference, insulin, glucose, total cholesterol, triglycerides, LDL-C, and serum cortisol and urinary free cortisol). A level of significance of $P < 0.05$ was required to enter the model (**Table 5**).

At Step 1, urinary 24 hrs norepinephrine entered the model and, together with age and gender, accounted for approximately 26% of the variability in chronotype score. At Step

2, the addition of RDI increased the variability in chronotype score accounted for by the model to 34%. Thus, the resulting final model retained two significant predictors, 24 hrs-urinary norepinephrine and RDI, which were both strongly related in an inverse fashion to chronotype score.

Discussion

In this cohort of sleep-deprived obese subjects, eveningness was associated with an unhealthy eating pattern, characterized by eating later on both working and non-working days and a trend towards a decreased number of eating occasions with larger portion sizes. Evening types were also more likely to have sleep apnea than Morning types, had higher levels of stress hormones, and had a higher resting heart rate. In addition, moving from morningness toward eveningness scores was associated with an increase in BMI and a decrease in HDL-C.

Eating less frequently has been reported to be associated with higher BMI and increased risk of weight gain.[23] Two- *vs.* threedaily meals were less effective in decreasing satiety levels and in inducing fat oxidation in lean women under isocaloric conditions.[24] Similarly, when lean men used to consume four-daily meals were switched to eating three-daily meals for four wks, less fat was oxidized and fat mass increased.[25] Consistent with our observations, evening types consumed twice as many calories after 20:00, and had a higher BMI than control subjects, while not differing in total food dietary.[26] In a cross-over, nonrandomized study of weight loss, obese women lost more weight and had greater fat oxidation when they were assigned to consume two-thirds of their total caloric intake in the morning *vs.* the evening. However the evening pattern was associated with a larger loss of fat *vs.* fat free mass and there was an order effect that may have influenced the conclusion.[27] A dietary intake study reported that food intake in the late night was less satiating and resulted in greater total energy intake.[28] Interestingly, meals consumed after 20:00 in our study contained less protein, which may decrease the satiating effect of food, as proteins are thought to be more satiating than carbohydrate.[29] Our results are also in agreement with a Finnish study of approximately 4500 adult subjects with a mean BMI of 27 kg/m^2 reporting unhealthy dietary habits in Evening types.[30]

In our sample, eveningness was associated with lower HDL-C levels, and a trend towards higher BMI and neck circumference. In a study of lean participants, Evening types tended to have increased BMI.[12] Social jetlag (discrepancy between sleep times on working *vs.* non-working days) was directly related to BMI in overweight and obese individuals.[4] Interestingly, overweight/ obese individuals carrying the 3111C polymorphism of the CLOCK gene, which is associated with eveningness, were more resistant to weight loss.[31] In our study, in which the actual sleeping schedule was left to the individual subject, we found no differences in sleep

quality andsleepiness scores between chronotypes. This is in accord with a well-conducted polysomnography study of 12 Morning types and 12 Evening types, tested in the sleep laboratory according to their habitual sleep schedule in which there were no differences in subjective sleep quality, as well as in total sleep time by EEG.[8] We report here for the first time that sleep apnea was twice as prevalent in the Evening *vs.* the Morning types, independent of BMI and neck circumference. This novel finding has important implications, given the overall negative impact of sleep apnea on health.

In a large population-based, Finnish study, the FINRISK 2007 survey, sleep duration did not differ between chronotypes,[32] but Evening types complained more often of insomnia, used sleeping pills more often, and had more frequent nightmares. In addition, the prevalence of short sleepers was significantly greater in men than in women with the Evening chronotype.

In our study sleep duration by actigraphy did not differ between chronotypes either, but Evening types reported sleeping approximately 40 min longer during working days. Further, self-reported sleep duration in both chronotypes was longer than sleep duration by actigraphy and this difference was present on both working and non-working days. We cannot account for the discrepancy between sleep duration by diary and actigraphy. It may take longer for Evening types to fall asleep because they feel less sleepy and may display more active behavior before going to bed. As an internal validation of self-reported measures, participants reported sleeping longer during non-working than during working days. Of note, most of the epidemiological studies on the relationship between sleep and weight relied on self-reported measures.[3] It has been recognized in this field that self-reported sleep and sleep by actigraphy do not correspond closely to each other.

The longer sleep latency period observed in our study for the Evening type may also be due to another related contributory factor, the quality of light. During the waking hours Evening types are less exposed to light, especially in the 100 to 500 lux range, which is the average intensity of interior lights.[33] Light exposure before bedtime delays melatonin onset and may therefore lengthen the period that is needed to fall asleep.[34] Of note, active behavior directly influences the central clock in the hypothalamus and can phase delay it when displayed in the evening hours. This may also lengthen the period that is needed to fall asleep.[35] Under constant routine conditions Evening types are the most alert around 22:30 whereas Morning types reach their peak of alertness much earlier in the day, around 13:00.[14]

In our sample, mean sleep efficiency was similarly suboptimal (81%,) in Morning and Evening types. A German actigraphy study conducted in a large sample of young students reported a somewhat better sleep efficiency in Morning types (88%) than Evening types (84%). It is possible that we have observed a “floor” effect in our sample that may have zeroed and obscured chronotype differences in sleep efficiency.

The intriguing possibility that assortative mating, defined as the nonrandom mating of individuals with respect to phenotype and cultural factors, may play a role in chronotype. This has been proposed in a report of a positive relationship in a German sample of 84 couples.[36] Assortative mating, whether positive or not, has been recently listed as one of the 10 putative factors contributing to the obesity epidemic .[37] As Evening types tend to be heavier and this is often already evident at reproductive age, future research should determine whether assortative mating for either factor has additive *vs.* synergistic effects in the etiology of obesity.

Compared to Morning types, Evening types had 20–30% higher 24 hrs urinary levels of epinephrine and higher morning plasma ACTH levels, indicating an activation of the sympathoadrenal system. As further evidence, the 24 hrs urinary norepinephrine was also borderline significantly elevated. In agreement with a previous report,[11] resting heart rate was higher in Evening *vs.* Morning types. Evening types have altered heart rate variability and a reduced ability to cope with stress.[38] The fact that ACTH levels were associated with chronotype, points to alterations in the feedback mechanisms of the hypothalamicpituitary-adrenal system, which has been reported in patients with sleep apnea and obesity.[39,40] Sleep apnea increases urinary norepinephrine and plasma ACTH levels.[40,41] However, the trend for increased urinary norepinephrine levels in Evening types was independent from increased sleep apnea. In turn, the chronic hyperventilation syndrome, that is breathing too deeply and too rapidly, as well as spending more time in lighter sleep stages, both more prevalent in Evening than Morning types, are risk factors for sleep apnea.[42,43]

The mean chronotype score, 53.7 seemed to be slightly skewed towards eveningness; for example, a study of 2,526 New-Zealand adults of averagely 40 yrs of age reported a mean chronotype score of 58.1,[44] and a study of 526 French adults with an average age of 51 yrs, reported a mean score of 59.6.[45] It is not clear whether cultural and ethnic factors are at play here or whether this represents normal variability.

As indicated by twin studies, there is a clear genetic component to morningness-eveningness, which accounts for more than 50% of its total variance.[46] Albeit subjects tend to become more morning-oriented with age, the age component of the total variance is smaller than the genetic component.[46] As we have recently reviewed, electrification and exposure to artificial light have limited the opportunities of the natural, bright light to synchronize our internal clock to the 24 hrs day cycle.[47] The magnitude of the synchronizing effect of bright light is dependent on the internal clock as well.[48]

Some study limitations should be noted. The cross-sectional nature of this report did not allow establishment of causality. Hormone determinations at a single time-point may have missed endocrine differences between chronotypes in circadian rhythms. Endocrine rhythms shift by two to three hrs depending on chronotype.[7] ACTH, for example, has its acrophase around 10:00 and, since we typically obtained blood at 08:00, measurements in

Morning types were potentially closer to the acrophase.[49] Another limitation of this ancillary study is that the chronotypes were unevenly distributed, with 2/3 of subjects belonging to the Morning chronotype and 1/3 to the Evening chronotype. Finally, we did not perform polysomnography measurements.

In summary, in our sample of obese, sleep-deprived subjects, eveningness was associated with a trend towards higher stress hormones, higher resting heart rate, more sleep apnea, lower HDL-C levels, and a trend towards higher BMI. High BMI and low HDL-C levels predict cardiovascular morbidity.[50] Our findings have public health relevance for the large number of obese individuals who have social jetlag and are sleep-deprived. We advocate for a heightened awareness in the medical field, as well as in the society at large, of the importance of chronotype and of the negative consequences of "forcing" our internal clock. The clinical approach to obese individuals should take into account individual sleep habits and chronotype. A similar characterization should be performed in the industrial sectors that employ shift workers. Finally, controlled studies are needed to test the effects of exposure to bright light on behavior, endocrine and metabolic features in different chronotypes.

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PART III

Health outcomes of insufficient sleep

CHAPTER 10

Sleep extension improves neurocognitive functions in short sleeping obese individuals

Eliane A. Lucassen,[1,2] Paolo Piaggi,[3] John Dsurney,[1,4] Lilian de Jonge,[5] Xiongce Zhao,[6] Megan S. Mattingly,[5] Angela Ramers,[5] Janet Gershengorn,[5] Gyorgy Csako,[1,7] and Giovanni Cizza,[5] for the Sleep Extension Study Group

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1. Clinical Center, National Institutes of Health, Bethesda, United States.
2. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
3. Obesity Research Center, Endocrinology Unit, University Hospital of Pisa, Pisa, Italy
4. Center for Neuroscience and Regenerative Medicine, Bethesda, United States.
5. Section on Neuroendocrinology of Obesity, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
6. Intramural Research Program, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, United States.
7. Department of Laboratory Medicine, Clinical Center, National Institutes of Health, Bethesda, United States.

Abstract

Background: Sleep deprivation and obesity, are associated with neurocognitive impairments. Effects of sleep deprivation and obesity on cognition are unknown, and the cognitive long-term effects of improvement of sleep have not been prospectively assessed in short sleeping, obese individuals.

Objective: To characterize neurocognitive functions and assess its reversibility.

Design: Prospective cohort study. Setting: Tertiary Referral Research Clinical Center.

Patients: A cohort of 121 short-sleeping (<6.5 hrs/night) obese (BMI 30–55 kg/m^2) men and pre-menopausal women.

Intervention: Sleep extension (468 ± 88 days) with life-style modifications.

Measurements: Neurocognitive functions, sleep quality and sleep duration.

Results: At baseline, 44% of the individuals had an impaired global deficit score (*t*-score 0–39). Impaired global deficit score was associated with worse subjective sleep quality (P = 0.02), and lower urinary dopamine levels (P = 0.001). Memory was impaired in 33%; attention in 35%; motor skills in 42%; and executive function in 51% of individuals. At the final evaluation (n = 74), subjective sleep quality improved by 24% (P < 0.001), self-reported sleep duration increased by 11% by questionnaires (P < 0.001) and by 4% by diaries (P = 0.04), and daytime sleepiness tended to improve (P = 0.10). Global cognitive function and attention improved by 7% and 10%, respectively (both P = 0.001), and memory and executive functions tended to improve (P = 0.07 and P = 0.06). Serum cortisol increased by 17% (P = 0.02). In a multivariate mixed model, subjective sleep quality and sleep efficiency, urinary free cortisol and dopamine and plasma total ghrelin accounted for 1/5 of the variability in global cognitive function.

Limitations: Drop-out rate.

Conclusions: Chronically sleep-deprived obese individuals exhibit substantial neurocognitive deficits that are partially reversible upon improvement of sleep in a non-pharmacological way. These findings have clinical implications for large segments of the US population.

Trail registration: www.ClinicalTrials.gov NCT00261898. NIDDK protocol 06-DK-0036

Introduction

According to the latest US surveys, 36% of the adult population is obese[1] and one third of adults report sleeping less than 6 hrs per night, substantially less than the recommended 7–9 hrs sleep per night.[2] Chronic sleep deprivation and obesity may be related in a bidirectional fashion, and they have similar consequences, including hypertension, diabetes, and cardiovascular disease.[3,4] In addition, obesity and sleep deprivation have been linked to cognitive deficits.

Obese individuals exhibit deficits in executive functions, including mental flexibility, planning, problem solving, and display impulsivity,[5-9] and tend to favor immediate reward *vs.* long term gain.[10] Possibly, the "higher cortical functions" that control learning and executive function are no longer appropriately inhibiting feeding behavior in obese individuals. In turn, altered eating behavior in obesity may cause neurocognitive dysfunction; for example a high-fat or high-carbohydrate meal can disrupt hippocampal function.[11]

Acute sleep deprivation predominantly interferes with attention and memory, while performance on more complex tasks is relatively intact.[12] One night of total sleep deprivation impaired attention in lean male volunteers,[13] and habitual short sleep decreased memory function in adolescents.[14] A meta-analysis reported that sleeping less than 30 hrs per week caused severe impairment in the clinical performance of medical care providers.[15] In addition, chronic sleep deprivation exacerbates symptoms of Attention Deficit Hyperactivity Disorder (ADHD).[16] Furthermore, sleep extension can improve declarative memory in adolescents.[17] Interestingly, six days of partial sleep restriction increased the activation of food reward pathways –a putative pathway leading to weight gain in chronically sleepdeprived individuals.[18]

Cognitive performance has never been evaluated in a sample of individuals who are both obese and chronically sleep deprived. Our goal was to characterize neurocognitive functions in this population and to prospectively assess reversibility of possible deficits with sleep extension achieved in a non-pharmacological way under real-life conditions.



Methods

Ethics statement

The NIDDK Institutional Review Board approved the protocol and each individual gave written informed consent. This research was conducted according to the principles expressed in the Declaration of Helsinki.

Study design and individuals

The Sleep Extension Study was a randomized, controlled, prospective study of sleep extension in chronically sleep-deprived obese individuals. Details have been provided elsewhere.[19] In brief, individuals were recruited by advertising for men and premenopausal women aged 18 to 50 yrs with a body mass index (BMI) between 30 and 55 kg/m^2 who reported sleeping less than 6.5 hrs per night. Individuals self-identified their ethnicity as "black", "white", or "other".

Out of 121 individuals with baseline neuropsychological evaluation, 72 were randomized to the Intervention Group and 49 to the Comparison Group. Individuals in the Intervention Group were coached to increase sleep duration up to 7.5 hrs per night, following a personalized sleep plan. Strategies included implementation of consistent bedtime routine, avoiding caffeine, alcohol, heavy meals and exercise prior to bedtime, creating an environment conducive to sleep, controlling bedroom light and temperature. The Comparison Group was asked to continue the existing short sleep habits. Sleeping habits were reviewed approximately every two months.

Study measurements

(A) Neurocognitive battery.

The battery of tests was administered in approximately 1.5 hrs between 2 pm and 6 pm in the following consistent order:

1) Wechsler Abbreviated Scale of Intelligence (WASI) (Psychological Corporation 1999, San Antonio, TX, USA): a brief test derived from the WAIS-III to estimate full scale IQ.
2) Rey Complex Figure Test: assesses visuo-spatial construction, nonverbal memory, attention, and planning.[20]
3) California Verbal Learning Test (CVLT-II): a list learning task to evaluate verbal learning and memory.[21]
4) Grooved Peg Board Test (GPeg) (Lafayette Instrument Company, Lafayette, IN, USA): a test of fine motor, manual dexterity.
5) Wisconsin Card Sort-test (WCST): a measure of executive function, emphasizing abstract reasoning, ability to use feedback and to shift cognitive sets.[22]
6) Trail Making Test (TMT) parts A and B: tests visual attention and task switching that requires visual scanning, sequencing, and visual motor speed.[23]
7) Verbal Fluency Test (FAS): assesses phonemic fluency associated with executive functioning.[24]
8) Iowa Gambling Task (IGT): a computer-based program developed to assess decision-making in a test that emulates gambling with varied cost *vs.* payoff ratios.[25]

These tests were grouped into four cognitive ability domains: a) Memory (Rey Delayed Recall, CVLT-II Short and Long Delay), b) Attention (Rey Immediate Recall, CVLT-II Sum, TMT-A), c) Motor skills (GPeg), and d) Executive function (TMT-B, WCST, FAS, IGT). The same battery of tests was administered at baseline and at the final follow up visit, except the WASI, which was only administered at baseline. The time interval between administrations of the tests would not be likely to produce practice effects.[26]

(B) Anthropometrics and body composition.

Height was measured using a wall mounted stadiometer (SECA 242, SECA North America East, Hanover, MD, USA) and weight was measured using a stand-on-scale in a hospital gown to the nearest 1/10th of a kg (SR555 SR Scales, SR Instruments, INC, Tonawanda, NY, USA). Circumference measurements were done using a non-stretch measuring tape in triplicate to the nearest mm. Waist circumference was measured at the uppermost lateral border of iliac crest at the end of a normal expiration. If this site could not be determined, the maximum circumference was measured at or near the level of the umbilicus. Neck circumference was measured at the minimal circumference with the head in the Frankfurt Horizontal Plane.

Abdominal visceral fat deposits were assessed by CT scans at the L2–L3 and L4–5 levels using a HiSpeed Advantage CT/I scanner (GE Medical Systems, Milwaukee, WI, USA) and analyzed on a SUN workstation using the MEDx image analysis software package (Sensor System, Sterling, VA, USA). Hypertension was defined as a blood pressure of ≥140/90 mmHg, and metabolic syndrome was determined by the NCEP ATP-III criteria.[27]

(C) Subjective and objective sleep measures.

Sleep duration was assessed by two-week sleep diaries and by concomitant usage of wrist actigraphy (Actiwatch-64, Mini Mitter/Respironics/Philips, Bend, OR, USA) via recording gross locomotor activity in one-minute epochs. Sleep efficiency was the sleep time divided by the time spent in bed. The respiratory disturbance index (RDI; the number of (hypo) apneas per hr of sleep) was documented overnight by a portable validated screening device (Apnea Risk Evaluation System, Advanced Brain Monitoring Inc., Carlsbad, CA, USA).[28] Daytime sleepiness was assessed by the Epworth Sleepiness Scale (ESS), a validated 8-item questionnaire with scores ranging from 0 to 24, with higher scores representing greater daytime sleepiness.[29] The Pittsburgh Sleep Quality Index (PSQI) is a validated 21-item questionnaire that quantifies subjective sleep quality.[30] PSQI scores range from 0 to 21 and higher scores indicate worse sleep.

(D) Clinical laboratory assessments.

Plasma ACTH and serum cortisol were determined by immunochemiluminescence methods (Immulite 2000 and 2500, Siemens Health Diagnostics, Deerfield, IL, USA). Total plasma ghrelin was measured with a sandwich ELISA kit measuring both intact and des-octanoyl forms with the lowest level of detection at 50 pg/mL for total ghrelin (Millipore, Billerica, MA, USA). The intra- and inter-assay CV's were 1.96% and 7.8%, respectively. Urine catecholamines and urinary free cortisol (UFC) were measured in 24 h-collections with high-performance-liquid chromatography, and liquid chromatography- tandem mass spectrometry, respectively.

Statistical analysis

Normality was determined by Q-Q plots. For normally distributed variables and for skewed variables, mean and standard deviation (SD) and median and interquartile range (IQR; 25th and 75th percentile) were computed, respectively.

All raw tests scores (TMT, GPeg, FAS) were converted to demographically adjusted *t*-scores, using Heaton norms [31] or test-specific norms, ranging from 0 to 100 (mean (SD): 50 (10)). A *t*-score below 40 was considered impaired; 40–44 was below average; 45–54 was average; and ≥ 55 was above average. Individual Rey scores were used only if the figure was copied well (Rey Copy *t*-score >39).

Raw Global Deficit Scores (GDS) were calculated by adding penalty points for the impairment category per separate test (*t*-score <20 = 5 points; *t*-score 20 - 24 = 4 points; *t*-score 25 - 29 = 3 points; *t*-score 30 - 34 = 2 points; *t*-score 35 - 39 = 1 point; and *t*-score >39 = 0 points), and dividing this number by the number of tests analyzed per individual. Domain Deficit Scores (DDS) were similarly computed for the tests of the specific neurocognitive domain. Raw GDS/DDS were then transformed into *t*-scores.[31] The GDS/DDS were considered "impaired" if the *t*-score was <40. This cut-off value has 69% sensitivity, and 91% specificity for diagnosing impairment.[32]

Independent *t*-tests and Mann-Whitney U tests were used to compare characteristics between individuals with a GDS/DDS *t*-score <40 *vs.* a *t*-score of ≥40. Statistical tests used to compare changes between baseline and follow-up included paired Student *t*-tests and Wilcoxon tests for skewed variables.

A multivariate mixed model using both baseline and follow-up data determined which variables were associated with GDS, after accounting for repeated measurements. Additional mixed models were constructed with change in GDS between the baseline and final evaluation visit as the dependent variable to examine the effect of sleep and anthropometric characteristics. Due to some heterogeneity in the residuals and also based on the AIC, final models had an identity covariance structure. Models were corrected for baseline parameters and for change in BMI. Statistical significance was defined as $P < 0.05$.

Results

Baseline neuropsychological, demographic, anthropometric, sleep, and hormonal characteristics according to global deficit score and domain deficit score

The mean age of the 121 individuals with neuropsychological testing at baseline was 41.1 ± 7.0 yrs, most were women (76%) and black (60%). They had 15.8 ± 2.5 yrs of education, an IQ of 112.8 ± 12.0, and a BMI of 38.6 ± 6.4 kg/m^2. Sleep duration by diaries averaged 6 hrs and 25 min (385 ± 48 min/night), whereas sleep duration by actigraphy monitors was approximately 30 min shorter (356 ± 49 min/night). Seven percent of the individuals smoked, 13% had hypertension, and 26% had metabolic syndrome.

The mean GDS was 41.0 ± 7.0, interpreted as below average (**Fig. 1**). Memory was impaired in 33%, attention in 36%, motor skills in 42%, and executive function in 51% of the individuals (**Fig. 1**).

Approximately 44% of individuals had an impaired (0–39) *t*-score. These individuals had a worse sleep quality by PSQI, a lower urinary dopamine, and tended to have a lower urinary norepinephrine and a lower UFC (**Table 1**). Demographic and anthropometric characteristics of the two groups were similar. Specific features that differed at a p-level of <0.1 for each of the cognitive domains examined are also shown in **Table 1**.

Individuals with an impaired memory had significantly lower urinary dopamine, norepinephrine, and UFC levels and a tendency for a lower sleep efficiency. Individuals with impaired attention had lower sleep efficiency, and lower urinary dopamine, norepinephrine, and UFC levels. Participants with impaired motor skills had a lower sleep efficiency. There were no differences in baseline characteristics level for participants with impaired *vs.* unimpaired executive functions.

Changes between the baseline and final neuropsychological evaluations

Of the 121 original individuals with baseline neuropsychological evaluation, 74 individuals (34 from the Comparison Group and 40 from the Intervention Group; 51F/23M) had another neuropsychological evaluation 468 ± 88 days later (median 436, IQR 412–496 days) (**Table 2**). Group allocation and GDS category were similar between the 74 individuals who completed the study and the 47 individuals who did not (P = 0.836 and P = 0.240, respectively). Because there were no significant differences in neuropsychological functions between the Comparison and the Intervention Groups either at the initial or at the final evaluation, results from the two groups were combined together.

The following changes were observed: the GDS improved by approximately 7%; attention improved by 10%; memory and executive functions tended to improve by 7% and 5%, respectively, whereas motor skills did not change. Individual tests that improved over time included the Rey Delayed Recall (memory), Rey Immediate Recall and CVLT-Sum (attention), and the IGT (executive functions) (**Table 2**).

Table 1. Baseline characteristics of individuals according to Global Deficit Score and Domain Deficit Score categories.

	GDS		
	***t*-score 0-39 (*n* = 53)**	***t*-score 40-58 (*n* = 68)**	***p* value**
Demographic			
Age (yrs)	41.8 (6.3)	40.4 (7.5)	0.27
Sex (% female)	83	71	0.11
Race (White/Black/Other in %)	62/36/2	57/35/7	0.38
Education (yrs)	15.7 (2.3)	15.8 (2.7)	0.73
Smokers (%)	8	6	0.75
Hypertension (≥140/90mmHg) (%)	16	13	0.62
Metabolic syndrome (%)	26	30	0.63
Anthropometric			
BMI (kg/m^2)	37.8 (6.0)	38.9 (6.6)	0.34
Waist circumference (cm)	113.2 (12.4)	115.6 (13.7)	0.88
Neck circumference (cm)	38.6 (4.0)	39.9 (4.3)	0.71
Abdominal visceral fat (cm^3)	319 (152)	357 (172)	0.98
Sleep			
Sleep duration by PSQI (min)	334 (62)	338 (45)	0.73
Sleep duration by diary (min)	383 (43)	387 (52)	0.70
Sleep duration by actigraphy (min)	351 (47)	345 (50)	0.48
Percent sleep efficiency	79.0 (7.3)	80.4 (5.7)	0.27
RDI (events/hrs)	7 (2 – 18)	8 (3 – 13)	0.65
Daytime sleepiness (ESS score)	8.1 (4.8)	8.4 (4.4)	0.71
Subjective sleep quality (PSQI score)	8.9 (2.9)	7.7 (2.7)	**0.04**
Abnormal subjective sleep quality (PSQI score >4) (%)	93	76	**0.02**
Hormonal			
Plasma ACTH (pg/mL)	18.2 (13.0 – 26.1)	18.9 (14.3 – 27.4)	0.48
Serum cortisol (µg/dL)	9.5 (3.8)	9.5 (4.3)	0.99
Urinary dopamine (µg/24h urine)	239 (169 – 292)	276 (230 – 341)	**0.001**
Urinary epinephrine (µg/24h urine)	3.4 (2.1 – 4.9)	4.5 (2.3 – 6.6)	0.24
Urinary norepinephrine (µg/24h urine)	38.0 (28.0 – 53.0)	44.5 (35.0 – 59.0)	0.07
Urinary free cortisol (µg/24h urine)	17.3 (12.0 – 23.3)	19.5 (14.5 – 28.3)	0.07
Plasma total ghrelin (pg/mL)	303 (199)	255 (125)	0.12
	DDS		
	***Memory* *t*-score 0-39 (*n* = 40)**	***Memory* *t*-score 40-58 (*n* = 81)**	***p* value**
Sleep efficiency (%)	78.1 (6.7)	80.6 (6.2)	0.06
Urinary dopamine (µg/24h urine)	228 (146 – 310)	269 (228 – 325)	**0.006**
Urinary norepinephrine (µg/24h urine)	38.0 (26.0 – 52.0)	44.0 (35.0 – 35.0)	**0.04**
Urinary free cortisol (µg/24h urine)	14.5 (10.3 - 20.0)	20.8 (15.0 – 28.5)	**0.01**
	***Attention* *t*-score 0-39 (*n* = 43)**	***Attention* *t*-score 40-58 (*n* = 78)**	***p* value**
Sleep efficiency (%)	78.0 (6.9)	80.8 (6.0)	**0.02**
Urinary dopamine (µg/24h urine)	241 (183 – 294)	276 (223 – 338)	**0.01**
Urinary norepinephrine (µg/24h urine)	37.5 (23.8 – 53.0)	45.0 (36.0 – 59.0)	**0.02**
Urinary free cortisol (µg/24h urine)	16.0 (10.0 – 24.0)	19.0 (15.0 – 28.0)	**0.02**
	***Motor Skills* *t*-score 0-39 (*n* = 51)**	***Motor Skills* *t*-score 40-58 (*n* = 70)**	***p* value**
Sleep efficiency (%)	78.1 (7.4)	81.1 (5.4)	**0.02**

Values are means (SD) for normally distributed variables, percent, or median (interquartile range, IQR) for skewed variables. *t*-tests were performed for continuous variables (skewed variables were log transformed before computations) and Chi-squared tests were used for categorical variables. Abbreviations: BMI = Body Mass Index, PSQI = Pittsburgh Sleep Quality Index, RDI = Respiratory Disturbance Index, ESS = Epworth Sleepiness Scale, ACTH = Adrenocorticotropic Hormone. SI conversion factors: To convert ACTH to pmol/L, multiply values by 0.22; to convert cortisol to nmol/L, multiply values by 27.588; to convert dopamine to nmol/d, multiply values by 6.528; to convert epinephrine to nmol/d, multiply values by 5.459; to convert norepinephrine to nmol/d, multiply values by 5.911; to convert free cortisol to nmol/d, multiply values by 2.759; to convert total ghrelin to pmol/L, multiply values by 0.296.

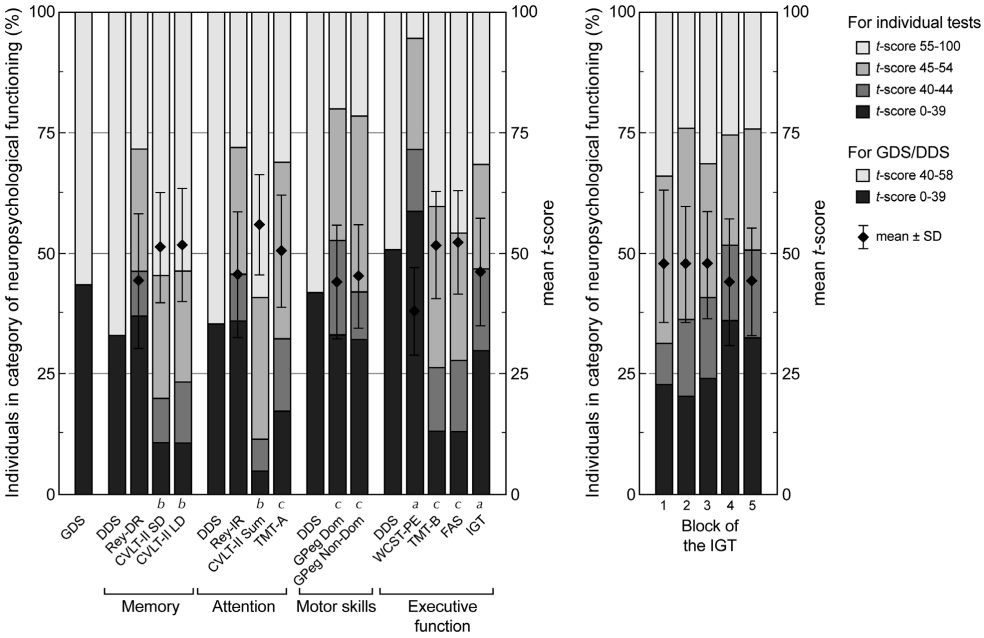


Fig. 1. Neurocognitive function at baseline. **Left**: Results of deficit scores and individual neurocognitive tests. **Right**: Results of the IGT per subsequent block of the test. The left vertical axis depicts the percentage of individuals in the following categories of neuropsychological function levels: impaired (*t*-score 0–39), below average (*t*-score 40–44), average (*t*-score 45–54), and above average (*t*-score 55–100). The right y-axis shows the mean ± SD of the *t*-scores for each separate test. Mean and SD for deficit scores are not shown, because these variables exhibited a skewed distribution. Memory was tested by Rey-DR, the CVLT-II SD and the CVLT-II LD. Attention was tested by the CVLT-II Sum and by the TMT-A. Motor skills were assessed by the GPeg Dom and the GPeg Non-Dom. Executive functions were tested by the WCST, the TMT-B and the IGT. For all *t*-score computations, age was taken into account. In addition, [a]yrs of education were taken into consideration for the *t*-scores for the WCST and IGT, and [b]gender was taken into account for the CVLT scores. [c]Education, race, and gender were all taken into account for *t*-scores of the TMT, GPeg, and FAS. Abbreviations: GDS = Global Deficit Score, DDS = Domain Deficit Score, Rey-DR = Rey Complex Figure Test Delayed Recall, CVLT-II SD = California Verbal Learning Test II Short Delay, CVLT-II LD = California Verbal Learning Test II Long Delay, CVLT-II Sum = California Verbal Learning Test II Sum Score, TMT-A = Trail Making Test Part A, TMT-B = Trail Making Test Part B, GPeg Dom = Grooved Peg Board Test Dominant Hand, GPeg Non-Dom = Grooved Peg Board Test Non-Dominant Hand, WCST = Wisconsin Card Sort-test, IGT = Iowa Gambling Task.

Subjective sleep quality improved by 24%, self-reported sleep duration increased by 11% by PSQI and by 4% by diaries, respectively, and sleepiness tended to improve. Serum cortisol increased by approximately 17% (**Table 3**). There were no significant changes in urinary dopamine, epinephrine and norepinephrine, UFC, and plasma total ghrelin. Of note, each of the characteristics shown in **Table 2** and **Table 3** were similar between "completers" and "non-completers" (data not shown) with the exception of UFC that was significantly lower in completers *vs.* non-completers (19.3 ± 12.6 *vs.* 24.8 ± 15.9 mg/24 hrs urine, $P = 0.038$).

Table 2. Changes in neurocognitive functions between the first and the final follow-up evaluation.

	Baseline (*n* = 74)[a]	**Final follow-up (*n* = 74)[a]**	***p* value**
GDS and DDS			
GDS	43.5 (9.2)	46.6 (9.0)	**0.001**
Memory DDS	47.7 (14.3)	50.9 (11.3)	0.07
Attention DDS	47.6 (11.3)	52.5 (10.2)	**0.001**
Motor Skills DDS	45.6 (14.6)	47.5 (14.7)	0.26
Executive function DDS	42.8 (9.8)	45.1 (11.0)	0.06
Specific neurocognitive test *t*-scores			
Rey Complex Figure Test			
Copy (*n* = 73)	44.4 (9.0)	43.3 (8.8)	0.26
Immediate Recall[b] (*n* = 49)	47.9 (12.7)	52.3 (12.4)	**0.002**
Delayed Recall[b] (*n* = 49)	45.6 (14.1)	51.1 (12.2)	**<0.001**
California Verbal Leaning Test			
Long Delay (*n* = 72)	52.1 (11.9)	53.5 (11.4)	0.12
Short Delay (*n* = 71)	51.5 (12.1)	53.1 (11.4)	0.08
Sum (*n* = 72)	55.4 (11.3)	57.9 (12.6)	**0.01**
Grooved Peg Board Test			
Dominant Hand (*n* = 69)	44.3 (11.8)	45.0 (11.9)	0.68
Non-Dominant Hand (*n* = 72)	44.8 (10.0)	46.1 (10.6)	0.17
Wisconsin Card Sort-test (Perseverative Errors; *n* = 72)	37.6 (9.1)	38.4 (7.7)	0.38
Trail Making Test part A (*n* = 73)	50.1 (12.0)	52.5 (12.7)	0.15
Trail Making Test part B (*n* = 72)	52.2 (11.6)	53.9 (11.8)	0.30
Verbal Fluency Test (*n* = 73)	52.4 (10.8)	52.9 (10.7)	0.60
Iowa Gambling Task (*n* = 48)	47.0 (11.2)	50.8 (11.5)	**0.04**

Values are means (SD). [a]Number of individuals per time point unless otherwise. [b]Scores for Immediate and Delayed Recall were only included if the Copy Score was at least 40, so that memory function was assessed in a more reliable manner. Abbreviations: GDS = Global Deficit Score; DDS = Domain Deficit Score.

Table 3. Changes in sleep and hormonal parameters between the baseline and final follow up evaluation.

Parameters	Baseline	Final follow-up	*p* value
Sleep parameters			
Sleep duration by actiwatch (min; *n* = 40)	356 (49)	360 (51)	0.51
Sleep duration by diary (min; *n* = 40)	388 (53)	405 (49)	**0.04**
Subjective sleep quality (PSQI score; *n* = 64)	8.0 (2.8)	6.1 (2.4)	**<0.001**
Sleep duration by PSQI (min; *n* = 64)	336 (60)	372 (72)	**<0.001**
Daytime sleepiness (ESS score; *n* = 63)	8.8 (4.8)	8.0 (4.5)	0.10
Anthropometrics			
BMI (kg/m^2; *n* = 74)	38.2 (6.7)	38.2 (6.9)	0.98
Waist circumference (cm; *n* = 74)	114 (14)	114 (15)	0.77
Neck circumference (cm; *n* = 73)	39.3 (4.3)	39.0 (4.2)	0.10
Total visceral fat (cm^3; *n* = 70)	352 (167)	351 (175)	0.95
Hormonal parameters			
Serum cortisol (µg/dL; *n* = 67)	8.7 (3.8)	10.2 (4.8)	**0.02**
Urinary dopamine (µg/24h urine; *n* = 69)	256 (199 – 317)	241 (188 – 331)	0.53
Urinary epinephrine (µg/24h urine; *n* = 69)	3.3 (2.0 – 4.8)	3.8 (2.3 – 5.5)	0.11
Urinary norepinephrine (µg/24h urine; *n* = 69)	38.5 (28.5 – 56.5)	40.0 (33.0 – 55.5)	0.35
UFC (µg/24h urine; *n* = 69)	15.0 (10.0 – 21.0)	16.0 (10.0 – 26.0)	0.76
Plasma total ghrelin (pg/mL; *n* = 67)	268.8 (148.8)	292.1 (204.6)	0.32

Values are means (SD) for normally distributed variables and median (interquartile range, IQR) for skewed variables. *t*-tests were performed (skewed variables were log transformed before computations). Abbreviations: PSQI = Pittsburgh Sleep Quality Index, ESS = Epworth Sleepiness Scale, UFC = Urinary Free Cortisol. SI conversion factors: To convert cortisol to nmol/L, multiply values by 27.588; to convert dopamine to nmol/d, multiply values by 6.528; to convert epinephrine to nmol/d, multiply values by 5.459; to convert norepinephrine to nmol/d, multiply values by 5.911; to convert free cortisol to nmol/d, multiply values by 2.759; to convert total ghrelin to pmol/L, multiply values by 0.296.

Relationships between neurocognitive function by domain deficit score, obesity, sleep and urinary stress hormones

Five variables were included in the model, sleep quality by PSQI and sleep efficiency, and UFC, urinary dopamine and plasma total ghrelin, together accounting for 1/5 of the variability in global cognitive functions (**Table 4**). The effect size was of clinical significance; as an example, an improvement in sleep quality of 1 unit would improve the GDS by 0.64 units, whereas an improvement in sleep efficiency of 1 unit would improve the GDS by 0.25 units. Adjustment by age increased the accounted variability from 22% to 28% (**Table 4**), whereas adjustments by gender, or ethnicity did not substantially change the explained variability (data not shown). Each model included the group allocation and follow-up time as covariates.

Table 4. Mixed models for Global Deficit Score.

Dependent Variable: GDS	***Model A: unadjusted***	***Model B: adjusted by age***
Intercept	22.5 (8.3) **(*P* = 0.008)**	12.4 (9.2) (*P* = 0.18)
Goodness of fit	R^2 = 0.224 **(*P* < 0.001)**	R^2 = 0.280 **(*P* < 0.001)**
Subjective sleep quality (PSQI score)	−0.64 (0.24) **(*P* = 0.01)**	−0.70 (0.24) **(*P* = 0.005)**
Sleep efficiency (%)	0.25 (0.10) **(*P* = 0.01)**	0.23 (0.10) **(*P* = 0.02)**
UFC (μg/24h urine)	0.09 (0.05) (*P* = 0.09)	0.10 (0.05) (*P* = 0.06)
Urinary dopamine (μg/24h urine)	0.02 (0.01) **(*P* = 0.001)**	0.03 (0.01) **(*P* < 0.001)**
Plasma total ghrelin (pg/mL)	−0.01 (0.01) (*P* = 0.07)	−0.01 (0.04) **(*P* = 0.02)**
Group (0=Comparison, 1=Intervention)	−0.41 (1.56) (*P* = 0.79)	−0.66 (1.51) (*P* = 0.66)
Follow-up time (days)	0.0020 (0.0024) (*P* = 0.41)	0.0020 (0.0025) (*P* = 0.42)
Age (yrs)	–	0.27 (0.11) **(*P* = 0.02)**

Mean (SE) of the regression coefficient is depicted as well as *p* value. The statistical model was fit as follows: at first, five candidate variables were preselected based on physiology. These included hormones, anthropometric and body composition features, glucose metabolism and sleep features. These variables were screened in bivariate analyses with the GDS score as dependent variable, using a *p* value less than 0.2, in order to avoid rejecting potentially important variables. Of note, the variables included in the model were also checked for multicolinearity by using the Variance Inflation Factor (VIF) index and found to be unrelated to each other (highest VIF = 1.6 where a value of 5 or higher is the conventional threshold used for multicolinearity). Abbreviations: PSQI = Pittsburgh Sleep Questionnaire Index, UFC = Urinary Free Cortisol. SI conversion factors: To convert cortisol to nmol/L, multiply values by 27.588; to convert dopamine to nmol/d, multiply values by 6.528; to convert total ghrelin to pmol/L, multiply values by 0.2

Influence of sleep and anthropometric characteristics on change between baseline and final neuropsychological evaluations

Mixed models were contructed to evaluate the modulation of sleep and anthropometric characteristics on the change in GDS. In unadjusted models, sleep duration by diary and by actiwatch, and sleep efficiency associated significantly with a change in GDS in a positive manner (**Table 5**). These associations remained significant when corrected for baseline characteristics and change in BMI between the baseline and final evaluation (**Table 5**). According to the adjusted models, an increase in sleep duration of 10 minutes by diary or by actiwatch would result in a modest 0.2-point improvement of GDS, and 100-minute increases would result in a 2.3 and 2.6-point amelioration in GDS, respectively. For sleep efficienty, a 10% increase would result in a GDS of 1.5 points higher. Adjusted models for waist and neck circumference and total visceral fat were also constructed without other anthropometrics as covariates, which did not alter conclusions (waist circumference: *P* = 0.129; neck circumference: *P* = 0.417; visceral fat: *P* = 0.181).

Table 5. Mixed models for change in Global Deficit Score between baseline and final evaluation visits.

Dependent Variable: improvement in GDS	***Unadjusted models***	***Models adjusted by BMI, change in BMI, yrs of education, gender, race***
Sleep parameters		
Sleep duration by diary	0.022 (0.009) **(*P* = 0.02)**	0.023 (0.010) **(*P* = 0.03**)
Sleep duration by actiwatch	0.022 (0.010) **(*P* = 0.01)**	0.026 (0.009) **(*P* = 0.006**)
Sleep efficiency	0.124 (0.062) **(*P* = 0.05)**	0.147 (0.070) **(*P* = 0.04**)
Subjective sleep quality (PSQI score)	0.014 (0.166) (*P* = 0.93)	0.017 (0.173) (*P* = 0.92)
Daytime sleepiness (ESS score)	0.039 (0.103) (*P* = 0.70)	0.044 (0.106) (*P* = 0.68)
Antropometrics		
BMI	0.038 (0.067) (*P* = 0.57)	0.052 (0.069) (*P* = 0.45)
Waist circumference	0.063 (0.032) (*P* = 0.05)	0.097 (0.060) (*P* = 0.11)
Neck circumference	0.193 (0.107) (*P* = 0.07)	0.103 (0.218) (*P* = 0.64)
Total visceral fat	0.005 (0.003) (*P* = 0.06)	0.004 (0.004) (*P* = 0.27)

Mixed models with an identity covariance structure were fit with sleep parameters or anthropometrics as independents. Mean (SE) of the regression coefficient and *p* value is depicted for each sleep or anthropometric parameter. Abbreviations: PSQI = Pittsburgh Sleep Questionnaire Index, UFC = Urinary Free Cortisol.

Discussion

To our knowledge, this is the first prospective report of neurocognitive functions in short sleeping obese men and women. A large percentage of individuals displayed neurocognitive deficits of clinical relevance, even though this sample was otherwise relatively healthy, with few smokers and few individuals with hypertension. Furthermore, individuals with more accentuated deficits had worse sleep quality and sleep efficiency, and a distinct hormonal profile characterized by lower urinary catecholamines and lower UFC. In a multivariate model, approximately 1/5 of the variability in the GDS, a parameter known to be influenced by many different factors, was accounted for by sleep quality and efficiency, as well as UFC, urinary dopamine and plasma total ghrelin. When testing was repeated 14 to 15 months later, improvements in sleep achieved in a non-pharmacological fashion were accompanied by ameliorations in neurocognitive scores. Although alternate forms of neuropsychological tests, when available, are best for multiple administrations, there was an extended period between testing and alternate forms were not used in this study. The time interval between administrations of the tests would not be likely to produce practice effects of a magnitude or direction,[33] which would confound the results. Improvement in GDS score associated with several sleep parameters, but not with anthropometric parameters. Therefore, these findings are strongly suggestive of a contributory role of sleep deprivation for the cognitive deficits observed. More importantly, they indicate that some of these deficits may be ameliorated by sleep improvement.

Sleep duration as assessed by actigraphy monitors and sleep duration by diaries were somewhat discrepant in our study. At baseline, sleep duration by actigraphy was

approximately 30 min shorter than sleep duration by diaries. Sleep duration usually is estimated by self-reported sleep in epidemiological studies, by actigraphy in smaller studies, and by polysomnography (PSG) in clinical studies. No method is without limitations in determining sleep duration; even PSG, which is considered the "gold standard", may interfere with sleep duration the night of the testing because of its "invasiveness". The degree of difference and its direction, with longer sleep duration with diaries, was similar to what previously reported.[34] Of note, the high correlation (0.90) between actigraphy and PSG observed in lean healthy subjects may worsen in subjects with sleep apnea because of limb movements during hypocapnia.[35] Therefore, in a sample of obese subjects, many with sleep apnea, actigraphy may be less reliable than in lean subjects.

Obese subjects often have impairments in the executive domain,[5,8,10,11] while sleep deprivation decreases attention and impairs processing speed.[12,14,16,36] Compared to other studies of obese adults, our participants had worse scores on the TMT-B (all tests of the executive domain),[35] but similar scores at the IGT or the WCST.[8,10,37,38] Medical residents that habitually sleep less than six hrs were quicker on the TMT-A and TMT-B (attention and executive function, respectively), and scored better on the CVLT-LD (memory) than our participants.[38] As in these studies scores were not corrected for demographic characteristics, differences with our findings should be interpreted with caution.

Sleep quality was worst in participants with memory, attention and motor domains impairments. This is likely independent of sleep apnea, since the RDI was similar between participants with impaired *vs.* normal cognitive function. Deficits in memory and executive functions have been reported in men with sleep apnea.[39] Another comparison to our participants is patients with chronic non-restorative sleep, in which memory function on the Rey test was correlated with sleep efficiency.[40] Patients with restless leg syndrome who experience decreased sleep efficiency also have impairments in executive functions.[41]

Sleep loss affects attention span. Because executive functions are impaired in obese individuals, we hypothesize that the compensatory ability of brain areas devoted to attention may be limited in obese, sleep-deprived individuals, resulting in a larger impairment compared to non-obese, sleep-deprived individuals. In our cohort, we found no relationship between adiposity and neurocognitive functions. This lack of association may be due to a "floor effect" – after a certain degree of obesity, cognitive functions may no longer be affected. Obese individuals with a mean BMI of 51 kg/m^2 scored worse than our individuals on the Rey-test and on the WCST, indicating that further cognitive impairment may occur when obesity is extreme.[5] The observed decrease in attention and decision-making ability in obese, sleep-deprived individuals may increase their risk of accidents. Medical residents, shift workers, and truck drivers are at higher risk for errors because of sleep deprivation. Thus, obese, sleep-deprived individuals may be similarly prone to accidents.

Impairments in memory and attention were associated with lower UFC, and lower 24 hrs urinary dopamine and norepinephrine. Brain dopaminergic and noradrenergic systems modulate cognition.[42,43] Since the stress response has a U-shaped curve, suboptimal levels of stress hormones may impair cognitive functions.

Plasma levels of the appetite stimulant ghrelin were inversely related to the GDS in our study participants (**Table 4**). This effect would be however of a modest clinical size: a 50-point increase in ghrelin would translate to only a decrease of 0.5 point in GDS. This finding may be of mechanistic interest, as the ghrelin system, in addition to its known role on appetite control, is involved in in neuroprotection, learning, and memory consolidation.[44]

Study merits included the prospective, long-term characterization of a large cohort assembled ad hoc. Sleep duration was assessed with several complementary methods. However, the study design did not allow for discerning how much of the cognitive deficits could be attributed to chronic sleep deprivation *vs.* obesity. The presence of a group of lean, sleep-deprived individuals, and a group of obese, non-sleep deprived would have allowed determination of the effect of obesity *vs.* sleep deprivation on cognitive functions. Finally, loss to follow-up was approximately 40% in this challenging study, which is what is usually observed in prospective studies of obese subjects.

In summary, this is the first demonstration of sleep extension in a real life situation in chronically sleep-deprived obese individuals. This population exhibited neurocognitive deficits that were partially reversible; self-reported sleep quality, duration and sleepiness, all improved to a clinically meaningful extent as well. These improvements were achieved in a non-pharmacological way in a real life situation and were sustained over a long time. Approximately 86 million adult Americans are obese, 40 million US workers report an average sleep duration of <6 hrs according to the CDC. Our findings have clinical implications for vast segments of the US population with obesity, and sleep deprivation. Further research is needed to dissect the relative role of chronic sleep deprivation *vs.* obesity on cognitive functions. Prospective studies of obese individuals undergoing bariatric surgery may be warranted.

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CHAPTER 11

The association between sleep and musculoskeletal parameters in middle-aged men and women: the NEO study

Eliane A. Lucassen,[1] Renée de Mutsert,[2] Saskia le Cessie,[3] Natasha M. Appelman-Dijkstra,[4] Frits R. Rosendaal,[2] Diana van Heemst D,[5] Martin den Heijer,[2,6] and Nienke R. Biermasz,[4] for the NEO study group

In preparation.

1. Laboratory of Neurophysiology, Department of Molecular Cell Biology, Leiden University Medical Center, Leiden, the Netherlands.
2. Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, the Netherlands.
3. Department of Medical Statistics, Leiden University Medical Center, Leiden, the Netherlands.
4. Department of Endocrinology, Leiden University Medical Center, Leiden, the Netherlands.
5. Department of Gerontology and Geriatrics, Leiden University Medical Center, Leiden, the Netherlands.
6. Department of Endocrinology, VU University Medical Center, Amsterdam, the Netherlands.

Abstract

Sleep deprivation has detrimental metabolic consequences. Results from studies linking sleep parameters to osteopenia or sarcopenia are scarce and have been inconsistent. Osteopenia and sarcopenia usually occur together and both reflect aging and increased risk of fractures and disease. We therefore aimed to examine the associations of sleep parameters with both osteopenia and sarcopenia, taking the influence of sex and menopause into account.

This is a cross-sectional analysis of 915 participants (45-65 years, 56% women, BMI 26 ± 5kg/m^2) in the Netherlands Epidemiology of Obesity (NEO) study, a population-based cohort study. Sleep duration, quality, and timing were assessed with the Pittsburgh Sleep Quality Index (PSQI); bone mineral density (BMD) and relative appendicular muscle mass (RASM) were measured by DXA scans. Linear and logistic regressions were performed to associate sleep parameters to BMD, RASM, osteopenia and sarcopenia.

After adjustment for confounding factors, one point increase in total PSQI score (**OR and 95% CI, 1.09, 1.03-1.14**), declined self-rated sleep quality (**1.76, 1.03-3.01**), and sleep latency (**1.18, 1.06-1.31**), and a one hour later sleep timing (**1.51, 1.08-2.11**), but not sleep duration (**1.05, 0.90-1.23**), were associated with osteopenia. Total PSQI score (**1.10, 1.02-1.19**) was also associated with sarcopenia and sleep latency (**1.14, 0.99-1.31**) and later mid-sleep time (**1.54, 0.91-2.61**) tended to associate with sarcopenia. Associations were somewhat stronger in women and varied per menopausal status. The association of sleep parameters and osteopenia was independent of RASM, and the association of sleep and sarcopenia was independent of BMD.

These results imply that decreased sleep quality and a later sleep timing are risk factors for osteopenia and sarcopenia in middle aged individuals. Further research is needed to assess whether improvement of sleep can prevent premature loss of bone and muscle mass.

Introduction

In modern society, more than one in three Americans report unintentionally falling asleep during the daytime, implying that insufficient sleep is an important public health concern.[1] Sleep deprivation has profound effects on health, including increased cardiovascular disease, diabetes, and obesity, which may be caused by alterations in sympatho-vagal balance, increased evening cortisol levels, pro-inflammatory changes, and/or decreased levels of growth hormone.[2] These effects are also observed in studies that selectively reduced sleep quality without affecting total sleep duration.[3,4] Additionally, sleep timing may impact health, as evening types generally have higher levels of catecholamines and cortisol.[5,6]

These endocrine, neural and inflammatory alterations mentioned above have the potential to negatively affect the musculoskeletal system. Recent studies suggest that insufficient sleep is associated with a decline in musculoskeletal parameters, although outcomes have been inconsistent.[7,8] Studies have reported positive,[9,10] negative,[11,12] or U-shaped[13] associations between sleep duration and bone mineral density (BMD), or observed no association.[7] One small study observed a negative association between sleep duration and skeletal muscle mass.[8] Self-reported sleep quality has been associated with an increased risk of osteoporosis[14] and fractures,[15,16] but in these studies sleep quality was assessed by one single question. Other studies observed that individuals with a later sleep timing were at increased risk of osteoporosis (BMD ≥2.5 SD below young reference population)[10,17] and sarcopenia (low muscle mass).[18]

Osteopenia and sarcopenia are prevalent conditions, with 22% and 36% of middle-aged men and 36% women classifying as osteopenic[19] and 27% and 35% of middle-aged men women as sarcopenic.[20] These frequently concurrent conditions become more prevalent with age and enhance each other's negative effects on health.[21] Fracture risk, for instance, increases more dramatically with age than would be estimated based on the corresponding decline in BMD alone,[22] likely largely due to a simultaneous age-related decline in muscle mass.[23-25] Up to now epidemiological studies have not associated sleep parameters with BMD and muscle mass simultaneously, while both parameters are important for musculoskeletal health and influence each other. [23-25] In this study, we aimed to examine associations of sleep duration, sleep timing and sleep quality with osteopenia and sarcopenia in a population based cohort study, and to what extent these associations differ between men and women and by menopausal status.



Materials and methods

Study design and study population

The Netherlands Epidemiology of Obesity (NEO) study is a population-based prospective cohort study that included 6,671 participants between September 2008 and September 2012. This study was designed to investigate pathways leading to disease in overweight

and obese individuals. Men and women aged between 45 and 65 years with a self-reported BMI of 27 kg/m^2 or higher from the city of Leiden and its surrounding municipalities (the Netherlands) were eligible to participate and were recruited via registries, general practitioners, and advertisements. In addition, all inhabitants aged between 45 and 65 years from one municipality (Leiderdorp) were invited irrespective of their BMI, allowing for a reference distribution of BMI. Participants were invited for a baseline visit at the NEO study center of the Leiden University Medical Center (LUMC) after an overnight fast.
The present study is a cross-sectional analysis of data obtained at the baseline visit. Prior to this visit, all participants completed questionnaires with demographic, lifestyle, and clinical information, in addition to a questionnaire on sleep behaviour. At the baseline visit, anthropometric measurements and fasting blood samples were obtained in the morning after participants fasted for at least 10 hours. Furthermore, a random subsample of 915 participants underwent dual energy X-ray absorptiometry (DXA) measurements between 10:00 and 12:30, assessing bone mineral densitometry (BMD), body fat mass and lean body mass. For the present analysis, we only included participants with DXA measurements. Individuals with bedtimes between 5am and 8pm were excluded from sleep timing analyses (n = 4). Study design and population have been described more extensively previously.[26]
This study was approved by the medical ethics committee of the Leiden University Medical Center (LUMC) and all participants provided written informed consent.

Data collection
Ethnicity, education, menopausal status, medication use, and past fractures were self-reported. Education was considered high if participants highest level of education was higher vocational school, university, or post-graduate education. Menopausal status was divided into three categories (pre-, peri-, and postmenopausal) based on information on oopho- or hysterectomy and/or self-reported menopausal status. Women who reported a hysterectomy were classified according to their age (premenopausal if <46 years, perimenopausal if 46 to 55 years, and postmenopausal if ≥55 years). Women who did not report menopausal status and were 58 years or older were classified as postmenopausal. In addition, all use of medication in the month preceding the study visit was recorded.

BMI was calculated from the height, measured with a fixed and calibrated tape measure, and the weight, measured without shoes and one kilogram was subtracted to correct for the weight of clothing. Waist and hip circumference were measured mid-way between the lower costal margin and the iliac crest and at the maximal circumference of the buttocks, respectively.

Habitual alcohol intake in grams per day was assessed using a semi-quantitative food frequency questionnaire (FFQ), which has been validated in the Dutch population,[27] and calculated from the 2011 version of the Dutch food composition table.[28]

Participants reported the frequency and duration of their usual physical activity in the past 4 weeks on the Short Questionnaire to Assess Health-enhancing physical activity (SQUASH), a method previously validated in the Dutch population.[29,30] We calculated the energy expended during physical activity in leisure time in hours per week of metabolic equivalents (MET-hr/week).

Serum vitamin D 25(OH) concentrationswere measured with LC-MSMS calibrated 2nd generation electrochemoluminescence immunoassay (ECLIA).

Sleep measurements

The Pittsburg Sleep Quality Index (PSQI) was completed at home prior to the baseline visit.[31] It includes questions about the average estimated duration of actual sleep and the usual bedtime and risetime during the past month. We defined the mid-sleep time as the clock time when half of the total sleep duration was completed, calculated from the usual bedtime and risetime. The questionnaire also contained questions concerning self-rated sleep quality ("very good", "fairly good", "fairly bad", or "very bad"), sleep latency (range: 0 – 6 points) and sleep duration. The total PSQI score ranges from 0 to 21, with higher scores indicating a worse sleep quality.

DXA bone and muscle measurements

Absolute BMD values were obtained for the lumbar spine (L1-L4) and the proximal femur regions (Hologic Discovery A, Tromp Medical BV, Castricum, the Netherlands). Mean vertebral (∑ L1-4 BMD / 4) and mean hip BMD (left BMD + right BMD / 2) were calculated for each individual. When the left or right hip measurement was missing (*n* = 10; 1.1%), we used the available side for analysis. T-scores were calculated based on the number of standard deviations below the BMD of a younger reference population of the NHANES III taking sex and ethnicity into account.[32] A t-score between -1 and -2.5 in either hip or vertebrae was considered as osteopenic, and -2.5 or lower as osteoporotic.[17] Whole body fat mass (kg) was assessed from the total body DXA scans.

The relative appendicular skeletal muscle mass (RASM) was calculated by summing the lean mass of the arms and legs and was corrected for body weight by division by the whole body mass as measured by DXA. Sarcopenia was defined as a RASM of one standard deviation below the RASM of a young reference population (RASM below 29.9% and 25.1% for men and women, respectively).[33]

Statistical analysis

Stata version 12.1 was used for analyses (StataCorp LP, TX, US). In the NEO study, individuals with a BMI of 27 kg/m^2 or higher were oversampled. To correctly represent associations in the general population,[34] adjustments for the oversampling of individuals with a BMI ≥ 27

kg/m^2 were made. This was done by weighting all participants towards the BMI distribution of participants from the Leiderdorp municipality,[35] whose BMI distribution was similar to the BMI distribution of the general Dutch population.[36] All results were based on weighted analyses. Consequently, the results apply to a population-based study without oversampling of participants with a BMI ≥ 27 kg/m^2.

Baseline characteristics of the weighted study population are given in mean (SD), median (interquartile range) for skewed variables, or as proportion of the study population. Pearson correlations between muscle and bone parameters were performed. Correlations between categorical parameters were assessed with Chi square tests. We performed linear regression analyses to examine the associations between sleep parameters and bone and muscle mass, expressed in mean differences with 95% confidence intervals (CI). In addition, we performed logistic regression analyses to calculate odds ratios and 95% CI for osteopenia and sarcopenia associated with the sleep parameters. Crude models, models adjusted for age, sex and total body fat, and models additionally adjusted for ethnicity, education, alcohol intake, physical activity, concentrations of vitamin D, season of the year, usage of systemic corticosteroids and bisphosphonates, and for menopausal status in women) were constructed. Finally, to examine whether sleep parameters associated with osteopenia and sarcopenia independent of muscle mass and BMD, respectively, we additionally adjusted the models of osteopenia for muscle mass and models of sarcopenia for BMD. We examined whether associations differed in men and women and by menopausal status in women by testing interaction terms with sex and also stratified all analyses by sex and menopausal status.

Results

Of 6,671 middle-aged men and women of the NEO cohort, 915 participants who underwent DXA were included in present analysis. Participants were 59 ± 4 years of age (range: 45-65 years), had a mean BMI of 25.5 ± 4.6 kg/m^2, and 56% were women. Participants that underwent DXA scans averagely had a 9% higher BMI and had a 8% lower PSQI score than other NEO participants.

Baseline characteristics of participants are presented in **Table 1**, stratified by sex. Men smoked more often, had a higher BMI, but had less body fat (as measured by DXA) than women. BMD in the spine and the hip and RASM were higher in men. The occurrence of osteopenia and osteoporosis did not differ between men and women, while the prevalence of sarcopenia was lower in men. Men had less sleep latency, but lower sleep quality measures and later sleep times than women.

Table 1: Baseline characteristics of participants of the Netherlands Epidemiology of Obesity study, aged 45 to 65 years and stratified by sex.

	Men	Women
General characteristics		
Age (years)	56 (6)	55 (6)
Ethnicity (% Caucasian)	95	93
Current smoker (%)	16	10
Former smoker (%)	44	43
Education (% high)[1]	50	46
BMI kg/m^2	27 (4)	25 (5)
Whole body fat mass (kg)	24 (7)	27 (9)
Whole body fat (% of total body weight)	26 (5)	36 (6)
Premenopausal (%)	N.A.	20
Perimenopausal (%)	N.A.	22
Postmenopausal (%)	N.A.	58
Age of menopause	N.A.	50 (4)
Habitual alcohol consumption (g/day)	14 (6 – 26)	7 (1 – 14)
Physical activity (MET-h/week)	26 (14 - 46)	27 (17 - 49)
Past fractures (%)	41	31
Vitamin D 25(OH) (nmol/l)	58 (25)	63 (28)
Usage vitamin D supplements (%)	4	7
Usage bisphosphonates (%)	0	1
Usage calcium supplements (%)	2	10
Usage systemic corticoids (%)	1	1
Usage sleeping medication (%)	4	5
Usage benzodiazepines (%)	2	4
Usage cyclopyrrolones (%)	1	1
Usage melatonin (%)	0.4	1
Usage homeopathic sleeping medication (%)	0.2	0
Sleep characteristics		
PSQI total score (range: 0 – 21)	4.1 (2.6)	5.1 (3.2)
Self-rated sleep quality “very good” (%)	32	21
Self-rated sleep quality “fairly good” (%)	55	62
Self-rated sleep quality “fairly bad” (%)	13	15
Self-rated sleep quality “very bad” (%)	1	1
Sleep latency (range: 0 - 6)	0 (0 - 1)	1 (0 - 3)
Sleep latency (min)	10 (5 - 15)	15 (10 - 30)
Usual bedtime (hh:mm (min))	23:22 (53)	23:10 (46)
Usual mid-sleep time (hh:mm (min))	3:50 (30)	4:05 (31)
Usual rise time (hh:mm (min))	7:03 (66)	7:21 (56)
Sleep duration (hrs)	7.0 (1.0)	7.1 (1.0)
Bone and muscle characteristics		
BMD spine (g/cm^2)	1.1 (0.1)	1.0 (0.1)
BMD hip (g/cm^2)	1.0 (0.1)	0.9 (0.1)
BMD spine t-score	-0.4 (1.3)	-0.5 (1.3)
BMD hip t-score	-0.1 (0.8)	-0.3 (1.0)



Table 1: Continued

	Men	Women
Osteopenic in spine (%)	30	29
Osteopenic in hip (%)	13	24
Osteopenic in hip or spine (%)	35	40
Osteoporotic in spine (%)	4	6
Osteoporotic in hip (%)	0	0
Osteoporotic in hip or spine (%)	4	6
ASM (kg)	30 (4)	19 (3)
RASM (% of body mass)	33 (3)	27 (3)
Sarcopenic (%)	9	27

[1]high education: higher vocational school, university, or post-graduate education. BMI, body mass index; MET, metabolic equivalents; PSQI, Pittsburgh Sleep Quality Index; C1, Component 1; C2, Component 2, BMD, bone mineral density; ASM, appendicular skeletal muscle mass; RASM, relative appendicular skeletal muscle mass. Data are presented as mean (SD), median (25th-75th percentile) or percentage. Results were based on analyses weighted towards the BMI distribution of the general population (*n* = 915).

BMD in the spine and hip correlated with RASM (R^2 = 0.116, *P* = 0.002 and R^2 = 0.278, *P* < 0.001), but osteopenia did not correlate with sarcopenia (X^2 = 0.006, *P* = 0.85). The prevalence of sarcopenia was 29% for participants with a healthy BMD, 29% for those with osteopenia and 29% for those with osteoporosis. Of the participants with sarcopenia, 32% had osteopenia and 4% had osteoporosis.

Associations of sleep parameters with BMD and muscle mass

Table 2 shows the mean differences in BMD in the spine and hip and muscle mass (RASM) associated with one unit change in the sleep parameters. After adjustment for potential confounding factors, measures of sleep quality were weakly associated with BMD in the spine and hip. For example, per point higher PSQI score (range: 0 - 21, higher values represent worse sleep quality) BMD was 0.003 g/m^2 lower in the spine and hip, respectively (about 0.3% / PSQI unit of BMD of the average participant). With each point higher on sleep latency (range: 0 - 6 points, higher values represent longer sleep latency) BMD in the spine was 0.007 g/m^2 lower (0.7% per sleep latency unit of BMD of the average participant).

In analyses stratified for sex, higher PSQI score was only associated with lower BMD in women, and not in men (**Suppl. Table 1**). Likewise, poorer self-rated sleep quality associated with lower BMD in women only. Sleep duration was associated with lumbar spine BMD only in men: a one hour decline in sleep duration related to a 2% lower BMD.

In additional analyses, associations were stratified by menopausal status (**Suppl. Table 2**). Of all women, 20% were premenopausal, 22% were perimenopausal and 58% were postmenopausal. The associations between one point higher in PSQI score and change in BMD appeared to be of a somewhat larger effect size in pre- and perimenopausal (between 0.7% and 1.0% of BMD of average participants) women than in postmenopausal women. In perimenopausal women, one point higher on PSQI associated with a 0.4% lower RASM.

Table 2: Difference (95% CI) in BMD spine, BMD hip and RASM per unit change of sleep parameter in the whole cohort.

Sleep measure	BMD spine (g/cm²)	BMD hip (g/cm²)	RASM (% of total body weight)
Crude models			
PSQI score	**-0.005 (-0.008 - -0.001)****	**-0.006 (-0.009 - -0.003)****	**-0.057 (-0.088 - -0.027)****
Self-rated sleep quality			
Very good (reference)	-	-	-
Fairly good	**-0.025 (-0.049 - -0.001)*****	**-0.039 (-0.059 – -0.019)***	**-0.922 (-1.620 - -0.225)***
Fairly or very bad	-0.029 (-0.063 - -0.004)	-0.023 (-0.055 – 0.010)	**-1.202 (-2.180 - -0.225)***
Sleep latency score	**-0.012 (-0.019 - -0.006)****	**-0.013 (-0.019 - -0.013)****	**-0.627 (-0.810 - -0.444)****
Mid-sleep time (hrs)	**-0.036 (-0.057 - -0.015)****	**-0.049 (-0.067 - -0.007)****	**-0.644 (-0.822 - -0.465)****
Sleep duration (hrs)	**-0.011 (-0.021 - 0.000)***	**-0.010 (-0.020 - -0.001)***	**-0.125 (-0.214 - -0.035)****
Age-, sex-, and whole body fat mass-adjusted models			
PSQI score	**-0.004 (-0.007 - 0.000)***	**-0.003 (-0.006 - 0.000)***	-0.011 (-0.029 - 0.007)
Self-rated sleep quality			
Very good (reference)	-	-	-
Fairly good	-0.011 (-0.034 – 0.012)	-0.018 (-0.036 – 0.001)	-0.112 (-0.397 – 0.173)
Fairly or very bad	-0.022 (-0.053 – 0.009)	-0.022 (-0.034 – 0.021)	-0.081 (-0.479 – 0.317)
Sleep latency score	**-0.007 (-0.013 - -0.001)***	-0.002 (-0.008 - 0.003)	-0.070 (-0.155 - 0.014)
Mid-sleep time (hrs)	-0.014 (-0.035 - 0.006)	-0.012 (-0.028 - 0.004)	**-0.139 (-0.246 - -0.032)***
Sleep duration (hrs)	-0.006 (-0.016 - 0.004)	-0.004 (-0.012 - 0.005)	-0.031 (-0.080 - 0.019)
Maximally adjusted models			
PSQI score	-0.003 (-0.007 - 0.000)	-0.003 (-0.006 - 0.000)	-0.026 (-0.076 - 0.023)
Self-rated sleep quality			
Very good (reference)	-	-	-
Fairly good	-0.017 (-0.035 – 0.001)	0.012 (-0.035 – 0.011)	-0.165 (-0.442 – 0.112)
Fairly or very bad	-0.006 (-0.034 – 0.023)	-0.020 (-0.051 – 0.010)	-0.002 (-0.396 – 0.392)



Table 2: Continued

Sleep measure	BMD spine (g/cm²)	BMD hip (g/cm²)	RASM (% of total body weight)
Sleep latency score	**-0.007**	-0.003	-0.061
	(-0.014 - -0.001)*	(-0.008 - 0.002)	(-0.146 - 0.025)
Mid-sleep time (hrs)	-0.014	-0.010	-0.091
	(-0.035 - 0.006)	(-0.026 - 0.007)	(-0.344 - 0.162)
Sleep duration (hrs)	-0.008	-0.004	-0.027
	(-0.018 - 0.002)	(-0.013 - 0.005)	(-0.102 - 0.156)

Crude; age, sex, whole body fat mass adjusted; and maximally adjusted (age, sex, whole body fat mass ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates) models are shown. PSQI, Pittsburgh Sleep Quality Index; BMD, bone mineral density; RASM, relative appendicular skeletal muscle mass. Results were based on analyses weighted towards the BMI distribution of the general population (n = 915). Significant associations are bolded. *P < 0.05, **P < 0.01, ***P < 0.001.

Suppl. Table 1: Difference (95% CI) in BMD spine, BMD hip and RASM per unit change of sleep parameter stratified by sex.

Sleep measure	BMD spine (g/cm²)		BMD hip (g/cm²)		RASM (% of total body weight)	
	men	women	men	women	men	women
Crude models						
PSQI score	0.000	**-0.005**	0.001	**-0.005**	-0.089	-0.106
	(-0.005 - 0.006)	**(-0.010 - -0.001)***	(-0.004 - 0.006)	**(-0.008 - -0.001)***	(-0.187 - 0.009)	(-0.218 - 0.005)
Self-rated sleep quality score						
Very good (reference)	-	-	-	-	-	-
Fairly good	0.004	**-0.039**	0.004	**-0.054**	-0.056	-0.050
	(-0.029 – 0.036)	**(-0.073 - -0.005)***	(-0.029 -0.036)	**(-0.081 - -0.027)*****	(-0.623 – 0.510)	(-0.734 – 0.635)
Fairly or very bad	0.003	**-0.045**	0.022	-0.034	-0.140	-0.362
	(-0.045 – 0.051)	**(0.089 - -0.011)***	(-0.023 – 0.066)	(-0.075 – 0.007)	(-0.978 – 0.697)	(-1.302 – 0.579)
Sleep latency score	-0.002	**-0.011**	0.004	**-0.008**	-0.154	-0.073
	(-0.013 - 0.009)	**(-0.019 - -0.003)****	(-0.006 - 0.015)	**(-0.015 - -0.002)***	(-0.344 - 0.037)	(-0.257 - 0.112)
Mid-sleep time (hrs)	-0.005	**-0.037**	-0.014	**-0.033**	0.018	0.022
	(-0.038 - 0.027)	**(-0.066 - -0.009)****	(-0.039 - 0.011)	**(-0.057 - -0.009)****	(-0.010 - 0.047)	(-0.004 - 0.048)
Sleep duration (hrs)	-0.014	-0.005	-0.011	-0.005	0.073	0.190
	(-0.030 - 0.001)	(-0.019 - 0.009)	(-0.024 - 0.002)	(-0.017 - 0.008)	(-0.202 - 0.349)	(-0.091 - 0.472)
Age & whole body fat mass-adjusted models						
PSQI score	-0.001	**-0.005**	0.000	**-0.004**	-0.035	-0.039
	(-0.006 - 0.005)	**(-0.009 - 0.000)***	(-0.005 - 0.004)	**(-0.008 - 0.000)***	(-0.088 - 0.019)	(-0.108 - 0.030)
Self-rated sleep quality score						
Very good (reference)	-	**-**	-	-	-	-
Fairly good	0.003	-0.022	0.004	**-0.038**	-0.001	-0.202
	(-0.028 – 0.035)	(-0.054 – 0.011)	(-0.022 -0.030)	**(-0.063 - -0.013)****	(-0.366 – 0.364)	(-0.640 – 0.235)
Fairly or very bad	0.004	-0.038	0.017	-0.028	-0.232	-0.042
	(-0.043 – 0.050)	(-0.081 – 0.004)	(-0.024 – 0.058)	(-0.066 – 0.010)	(-0.724 – 0.259)	(-0.638 – 0.554)
Sleep latency score	-0.003	-0.006	0.003	-0.004	-0.111	-0.055
	(-0.014 - 0.008)	(-0.014 - 0.002)	(-0.008 - 0.013)	(-0.010 - 0.002)	(-0.234 - 0.012)	(-0.167 - 0.056)

Sleep measure	BMD spine (g/cm²)		BMD hip (g/cm²)		RASM (% of total body weight)	
	men	women	men	women	men	women
Mid-sleep time (hrs)	-0.004 (-0.037 - 0.028)	-0.023 (-0.050 - 0.004)	-0.003 (-0.028 - 0.022)	-0.019 (-0.040 - 0.002)	-0.035 (-0.454 - 0.383)	-0.101 (-0.457 - 0.256)
Sleep duration (hrs)	-0.013 (-0.028 - 0.002)	-0.003 (-0.016 - 0.010)	-0.006 (-0.019 - 0.007)	-0.003 (-0.015 - 0.008)	-0.002 (-0.189 - 0.185)	0.044 (-0.147 - 0.235)
Maximally adjusted models[1]						
PSQI score	0.001 (-0.005 - 0.007)	**-0.004 (-0.009 - -0.000)***	0.001 (-0.004 - 0.005)	**-0.006 (-0.008 - -0.001)***	-0.007 (-0.064 - 0.050)	-0.043 (-0.114 - 0.028)
Self-rated sleep quality score						
Very good (reference)	-	-	-	-	-	-
Fairly good	0.000 (-0.031 – 0.032)	-0.023 (-0.055 – 0.009)	0.002 (-0.024 – 0.028)	**-0.040 (-0.064 – 0.159)****	-0.015 (-0.371 – 0.341)	-0.379 (-0.800 – 0.041)
Fairly or very bad	0.001 (-0.043 – 0.057)	**-0.042 -(0.082 – -0.002)***	0.025 (-0.020 – 0.071)	-0.035 (-0.071 – 0.002)	-0.020 (-0.479 – 0.439)	0.073 (-0.679 – 0.532)
Sleep latency score	-0.002 (-0.013 - 0.009)	-0.007 (-0.015 - 0.001)	0.003 (-0.008 - 0.013)	-0.005 (-0.011 - 0.002)	-0.054 (-0.176 - 0.067)	-0.063 (-0.178 - 0.051)
Mid-sleep time (hrs)	-0.004 (-0.036 - 0.027)	-0.025 (-0.052 - 0.002)	-0.001 (-0.025 - 0.028)	-0.019 (-0.040 - 0.002)	0.035 (-0.332 - 0.402)	-0.181 (-0.505 - 0.144)
Sleep duration (hrs)	**-0.017 (-0.032 - -0.001)***	-0.005 (-0.017 - 0.008)	-0.008 (-0.022 - 0.005)	-0.003 (-0.014 - 0.008)	-0.003 (-0.174 - 0.168)	0.055 (-0.127 - 0.238)

Crude, age & whole body fat mass adjusted, and maximally adjusted (age, whole body fat mass ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates, and menopause) models are shown. [1]age, whole body fat mass, ethnicity, education, alcohol intake, physical activity, menopause. PSQI, Pittsburgh Sleep Quality Index; BMD, bone mineral density; RASM, relative appendicular skeletal muscle mass. Results were based on analyses weighted towards the BMI distribution of the general population (n = 915). Significant associations are bolded. *P < 0.05, **P < 0.01, ***P < 0.001.

Suppl. Table 2: Difference (95% CI) in BMD spine, BMD hip and RASM per unit change of sleep parameter stratified by menopausal status.

Sleep measures	BMD spine (g/cm²)			BMD hip (g/cm²)			RASM (% of total body weight)		
	premeno-pausal	perimeno-pausal	postmeno-pausal	premeno-pausal	perimeno-pausal	postmeno-pausal	premeno-pausal	perimeno-pausal	postmeno-pausal
Crude models									
PSQI score	-0.004 (-0.018 - 0.010)	-0.004 (-0.011 - 0.004)	-0.003 (-0.009 - 0.003)	-0.004 (-0.016 - 0.008)	**-0.007 (-0.014 - -0.001)***	-0.001 (-0.006 - 0.003)	-0.117 (-0.421 - 0.187)	**-0.231 (-0.379 - -0.083)****	-0.028 (-0.178 - 0.122)
Self-rated sleep quality score									
Very good (reference)	-	-	-	-	-	-	-	-	-
Fairly good	-0.011 (-0.072 – 0.051)	-0.018 (-0.067 – 0.031)	0.035 (-0.086 – 0.016)	-0.037 (-0.094 – 0.020)	-0.040 (-0.088 – 0.008)	**-0.047 (-0.081 - -0.013)****	0.458 (-1.022 – 1.939)	-0.323 (-1.79 – 1.14)	0.144 (-0.770 – 1.057)
Fairly or very bad	-0.046 (-0.163 – 0.071)	-0.019 (0.102 – 0.065)	-0.038 (-0.096 – 0.020)	0.003 (-0.066 – 0.071)	-0.036 (-0.128 – 0.057)	-0.023 (-0.070 – 0.025)	0.493 (-2.547 – 3.533)	-0.417 (-2.153 - 1.320)	-0.214 (-1.455 – 1.027)
Sleep latency score	-0.012 (-0.040 - 0.016)	-0.004 (-0.018 - 0.010)	-0.006 (-0.016 - 0.004)	-0.004 (-0.029 - 0.022)	-0.007 (-0.022 - 0.009)	-0.003 (-0.011 - 0.004)	-0.197 (-0.775 - 0.381)	-0.132 (-0.439 - 0.176)	0.048 (-0.185 - 0.282)
Mid-sleep time (hrs)	-0.045 (-0.101 - 0.011)	-0.021 (-0.078 - 0.036)	-0.026 (-0.065 - 0.012)	-0.047 (-0.103 - 0.009)	-0.017 (-0.068 - 0.034)	-0.022 (-0.051 - 0.008)	0.887 (-0.936 - 2.710)	0.021 (-0.843 - 0.884)	0.021 (-0.843 - 0.884)
Sleep duration (hrs)	-0.018 (-0.048 - 0.012)	-0.012 (-0.033 - 0.008)	-0.001 (-0.019 - 0.018)	-0.013 (-0.043 - 0.017)	-0.006 (-0.031 - 0.019)	-0.005 (-0.019 - 0.009)	0.056 (-0.657 - 0.769)	**0.447 (0.059 - 0.834)***	0.062 (-0.358 - 0.482)
Age- and whole body fat mass-adjusted models									
PSQI score	-0.006 (-0.019 - 0.006)	-0.006 (-0.014 - 0.001)	-0.003 (-0.009 - 0.003)	-0.007 (-0.017 - 0.003)	**-0.01 (-0.016 - -0.004)****	-0.001 (-0.006 - 0.004)	0.011 (-0.170 - 0.193)	**-0.136 (-0.217 - -0.056)****	-0.018 (-0.113 - 0.077)
Self-rated sleep quality score									
Very good (reference)	-	-	-	-	-	-	-	-	-
Fairly good	-0.006 (-0.064 – 0.051)	-0.003 (-0.049 – 0.044)	0.029 (-0.081 – 0.024)	-0.032 (-0.082 – 0.018)	-0.025 (-0.065 – 0.016)	**-0.042 (-0.077 - -0.008)***	0.145 (-0.826 – 1.120)	**-0.915 (-1.737 - -0.093)***	-1.660 (-0.699 – 0.367)
Fairly or very bad	-0.040 (-0.153 – 0.073)	-0.016 (-0.095 – 0.063)	-0.043 (-0.101 – 0.016)	0.010 (-0.061 – 0.080)	-0.035 (-0.118 – 0.049)	-0.029 (-0.076 – 0.017)	0.337 (1.605 – 2.279)	-0.491 (-1.457 – 0.474)	-0.089 (-0.862 – 0.684)

Sleep latency score	-0.012 (-0.038 - 0.014)	-0.004 (-0.018 - 0.010)	-0.004 (-0.014 - 0.006)	-0.003 (-0.026 - 0.021)	-0.007 (-0.021 - 0.006)	-0.001 (-0.008 - 0.006)	-0.102 (-0.453 - 0.250)	-0.119 (-0.306 - 0.067)	-0.028 (-0.168 - 0.112)
Mid-sleep time (hrs)	-0.038 (-0.088 - 0.011)	-0.019 (-0.071 - 0.032)	-0.022 (-0.061 - 0.017)	-0.039 (-0.083 - 0.005)	-0.016 (-0.061 - 0.029)	-0.015 (-0.042 - 0.013)	0.353 (-0.797 - 1.503)	-0.037 (-0.620 - 0.546)	-0.037 (-0.620 - 0.546)
Sleep duration (hrs)	-0.014 (-0.041 - 0.012)	-0.006 (-0.027 - 0.015)	0.001 (-0.018 - 0.019)	-0.009 (-0.036 - 0.018)	0.001 (-0.024 - 0.025)	-0.004 (-0.018 - 0.010)	-0.158 (-0.651 - 0.336)	0.221 (-0.026 - 0.468)	0.017 (-0.257 - 0.292)
Maximally adjusted models									
PSQI score	-0.009 (-0.019 - 0.001)	**-0.010 (-0.015 - -0.004)****	0.000 (-0.005 - 0.005)	-0.011 (-0.023 - 0.002)	**-0.007 (-0.015 - -0.000)***	-0.001 (-0.007 - 0.005)	-0.111 (-0.292 - 0.070)	**-0.132 (-0.224 - -0.039)****	-0.002 (-0.101 - 0.098)
Self-rated sleep quality score									
Very good (reference)	-	-	-	-	-	-	-	-	-
Fairly good	-0.015 (-0.075 – 0.044)	-0.006 (-0.050 – 0.038)	-0.016 (-0.066 – 0.034)	-0.035 (-0.083 – 0.014)	-0.025 (-0.067 – 0.017)	-0.034 (-0.068 - -0.000)	-0.330 (-1.180 – 0.519)	-0.803 (-1.667 – 0.062)	-0.142 (-0.701 – 0.418)
Fairly or very bad	-0.068 (-0.174 – 0.039)	0.051 (-0.114 – 0.013)	-0.022 (-0.079 – 0.034)	-0.023 (-0.080 – 0.034)	-0.058 (-0.131 – 0.016)	-0.019 (-0.065 – 0.026)	-0.239 (-2.165 – 1.572)	-0.502 (-1.545 – 0.541)	0.193 (-0.650 – 1.036)
Sleep latency score	-0.002 (-0.023 - 0.019)	-0.010 (-0.023 - 0.003)	0.000 (-0.008 - 0.007)	-0.013 (-0.038 - 0.012)	-0.007 (-0.020 - 0.006)	-0.002 (-0.012 - 0.008)	-0.141 (-0.467 - 0.186)	-0.130 (-0.330 - 0.069)	-0.012 (-0.163 - 0.138)
Mid-sleep time (hrs)	-0.035 (-0.075 - 0.006)	-0.021 (-0.069 - 0.027)	-0.019 (-0.046 - 0.007)	-0.029 (-0.089 - -0.032)	-0.036 (-0.089 - 0.018)	-0.027 (-0.064 - 0.011)	-0.232 (-0.559 – 1.024)	-0.054 (-0.769 - 0.662)	-0.362 (-0.826 - 0.101)
Sleep duration (hrs)	0.009 (-0.012 - 0.030)	-0.001 (-0.024 - 0.026)	-0.008 (-0.022 - 0.005)	0.007 (-0.026 - 0.040)	0.008 (-0.029 - 0.013)	-0.005 (-0.023 - 0.012)	0.173 (-0.305 - 0.652)	0.236 (-0.058 - 0.531)	-0.047 (-0.328 - 0.233)

Crude, age & whole body fat mass adjusted, and maximally adjusted (age, whole body fat mass, ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates) models are shown. PSQI, Pittsburgh Sleep Quality Index; BMD, bone mineral density; RASM, relative appendicular skeletal muscle mass. Results were based on analyses weighted towards the BMI distribution of the general population (n = 915). Significant associations are bolded. *P < 0.05, **P < 0.01, ***P < 0.001.



Sleep parameters and risk of osteopenia and sarcopenia

Odds ratios for osteopenia or sarcopenia associated with the sleep parameters are shown in **Fig. 1**. In our middle-aged study population, only 4% of men and 6% of women classified as osteoporotic, which prevented us to reliably determine the relationship between sleep parameters and osteoporosis. Of all participants, 38% had osteopenia in either the hip or the spine (35% of men and 40% of women) and 19% participants had sarcopenia (9% of men and 27% of women). Prevalence of osteopenia and sarcopenia differed per menopausal status. Of pre-, peri- and postmenopausal women, 26%, 28% and 50% had osteopenia, and 18%, 24%, and 31% had sarcopenia, respectively.

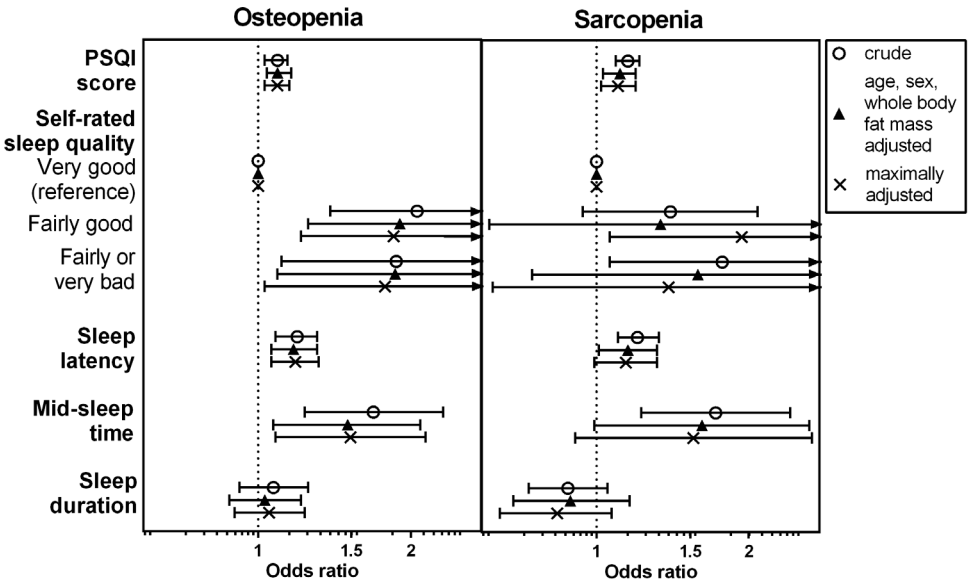


Fig. 1: OR (95% CI) for osteopenia and sarcopenia per unit change of sleep parameter. Crude; age, sex, whole body fat mass adjusted; and maximally adjusted (age, sex, whole body fat mass, ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, and usage of systemic corticosteroids and bisphosphonates) models are shown. PSQI, Pittsburgh Sleep Quality Index. Results were based on analyses weighted towards the BMI distribution of the general population (n = 915).

In the models adjusted for putative confounding factors, sleep quality and sleep timing were associated with higher odds of osteopenia and sarcopenia. Each point higher in PSQI score (indicating worse sleep quality) associated with a higher risk of osteopenia and sarcopenia by 9% and 11%, respectively. A “fairly bad” or “very bad” self-rated sleep quality associated with a 76% higher risk of osteopenia compared to a “very good” self-rated sleep quality. A longer sleep latency (range: 0 – 6) associated with a 18% and 16% higher risk for osteopenia and sarcopenia, respectively. A one hour later mid-sleep time associated with significantly higher risk for osteopenia by 51% and also tended to associate with higher

risk for sarcopenia. Sleep duration was not associated with osteopenia or sarcopenia in the whole study population.

When individuals with osteoporosis were included in the analyses and we associated sleep parameters with odds for osteopenia and osteoporosis, results were similar (**Suppl. Table 3**). Additionally, sleep parameters associated similarly with osteopenia in the spine and in the hip, although the mid-sleep time associated somewhat stronger with osteopenia in the hip than with osteopenia in the spine (**Suppl. Table 3**).

Suppl. Table 3: OR (95% CI) for osteopenia in the spine or hip (note: same values as depicted in **Fig. 1**), osteopenia or osteoporosis in the spine or hip, osteopenia in the spine, and osteopenia in the neck per unit change of sleep parameter.

Sleep measures	**Osteopenia spine or hip**	**Osteopenia or osteoporosis spine or hip**	**Osteopenia in spine**	**Osteopenia in hip**
Crude				
PSQI score	**1.09**	**1.08**	**1.06**	**1.09**
	(1.03 - 1.14)**	**(1.03 - 1.14)****	**(1.01 - 1.12)***	**(1.03 - 1.16)****
Self-reported sleep quality				
Very good (reference)	-	-	-	-
Fairly good	**2.03**	**1.95**	**1.78**	**2.38**
	(1.38-2.99)***	**(1.34 - 2.85)****	**(1.18 - 2.69)****	**(1.39 - 4.10)****
Fairly or	**1.85**	**1.73**	**1.86**	1.69
very bad	**(1.11-3.08)***	**(1.04 - 2.87)***	**(1.08 - 3.20)***	(0.82 – 3.50)
Sleep latency	**1.19**	**1.18**	**1.12**	**1.22**
score	**(1.08 - 1.30)****	**(1.07 - 1.29)****	**(1.01 - 1.23)***	**(1.10 - 1.36)****
Mid sleep	**1.67**	**1.62**	1.16	**2.01**
time (hrs)	**(1.23 - 2.28)****	**(1.19 - 2.19)****	(0.86 - 1.58)	**(1.35 - 2.97)****
Sleep	1.07	1.07	1.00	1.11
duration (hrs)	(0.92 - 1.25)	(0.92 - 1.24)	(0.85 - 1.18)	(0.91 - 1.36)
Age, sex, whole body fat mass corrected				
PSQI score	**1.09**	**1.09**	**1.07**	**1.09**
	(1.04 - 1.16)**	**(1.04 - 1.16)****	**(1.02 - 1.14)****	**(1.01 - 1.17)***
Self-reported sleep quality				
Very good (reference)	-	-	-	-
Fairly good	**1.98**	**1.92**	**1.75**	**2.36**
	(1.33 - 2.95)**	**(1.30 - 2.83)****	**(1.16 - 2.66)***	**(1.35 – 4.14)***
Fairly or	**1.93**	**1.82**	**1.92**	1.80
very bad	**(1.15 - 3.24)***	**(1.09 – 3.04)***	**(1.11 - 3.32)***	(0.86 - 3.78)
Sleep latency	**1.17**	**1.17**	**1.13**	**1.15**
score	**(1.06 - 1.30)****	**(1.06 - 1.30)****	**(1.02 - 1.26)***	**(1.01 - 1.31)***
Mid sleep	**1.49**	**1.44**	1.12	**1.62**
time (hrs)	**(1.07 - 2.06)***	**(1.04 – 2.00)***	(0.81 - 1.56)	**(1.04 - 2.54)***

Suppl. Table 3: Continued

Sleep measures	Osteopenia spine or hip	Osteopenia or osteoporosis spine or hip	Osteopenia in spine	Osteopenia in hip
Sleep duration (hrs)	1.03 (0.88 - 1.21)	1.03 (0.88 - 1.20)	0.98 (0.83 - 1.16)	1.05 (0.84 - 1.32)
Maximally adjusted				
PSQI score	**1.09 (1.03 - 1.15)****	**1.08 (1.02 - 1.15)****	**1.07 (1.01 - 1.13)***	**1.10 (1.01 - 1.19)***
Self-reported sleep quality				
Very good (reference)	-	-	-	-
Fairly good	**1.97 (1.31 - 2.97)****	**1.90 (1.27 - 2.84)****	**1.75 (1.14 - 2.68)***	**2.42 (1.36 - 4.30)****
Fairly or very bad	**1.97 (1.10 - 3.20)***	**1.76 (1.03 – 2.98)***	**1.88 (1.07 - 3.31)***	1.99 (0.93 – 4.26)
Sleep latency score	**1.18 (1.06 - 1.31)****	**1.18 (1.06 - 1.31)****	**1.13 (1.02 - 1.25)***	**1.17 (1.02 - 1.33)****
Mid sleep time (hrs)	**1.51 (1.08 - 2.11)***	**1.47 (1.05 - 2.05)***	1.11 (0.80 - 1.54)	1.58 (1.00 - 2.51)
Sleep duration (hrs)	1.05 (0.90 - 1.23)	1.05 (0.90 - 1.23)	0.99 (0.85 - 1.17)	1.02 (0.81 - 1.28)

Crude; age, sex, and whole body fat mass adjusted; and maximally adjusted (age, whole body fat mass, ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates) models are shown. PSQI, Pittsburgh Sleep Quality Index. Results were based on analyses weighted towards the BMI distribution of the general population (n = 915). Significant associations are bolded. *P < 0.05, **P < 0.01, ***P < 0.001.

Odds ratios of osteopenia and sarcopenia stratified by sex and menopausal status

In sex-stratified analyses most associations were stronger in women (**Fig. 2**). For PSQI and sleep latency, associations between men and women were only marginally different for osteopenia. For example, a point increase PSQI score associated with a 7% and 8% higher risk for osteopenia in men and women, respectively. Correspondingly, men and women with a one-point worse sleep latency score, had a 14% and 15% higher risk for osteopenia. For sarcopenia however, one point higher PSQI score associated with a 14% increased risk in women a longer sleep latency with a 19% higher risk, but these sleep parameters were not associated with sarcopenia in men. For self-rated sleep quality and mid-sleep time, associations were larger in women than in men for osteopenia and for sarcopenia. Similarly, women rating their sleep quality as “fairly or very bad” instead of “very good” had over a two-fold risk of osteopenia and sarcopenia, while this was not associated with musculoskeletal parameters in men. Sleep duration did not affect the risk of osteopenia or sarcopenia.

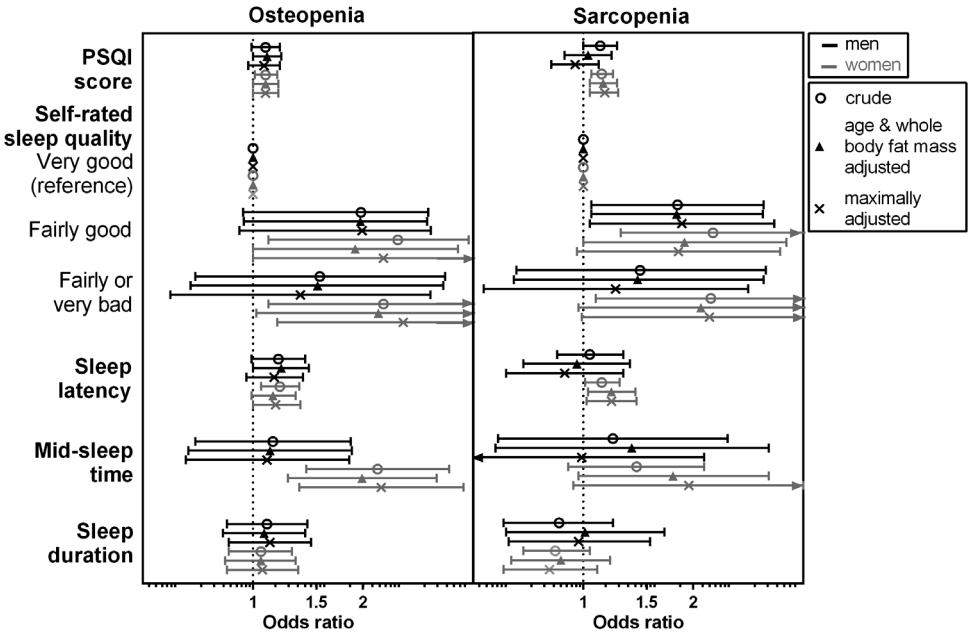
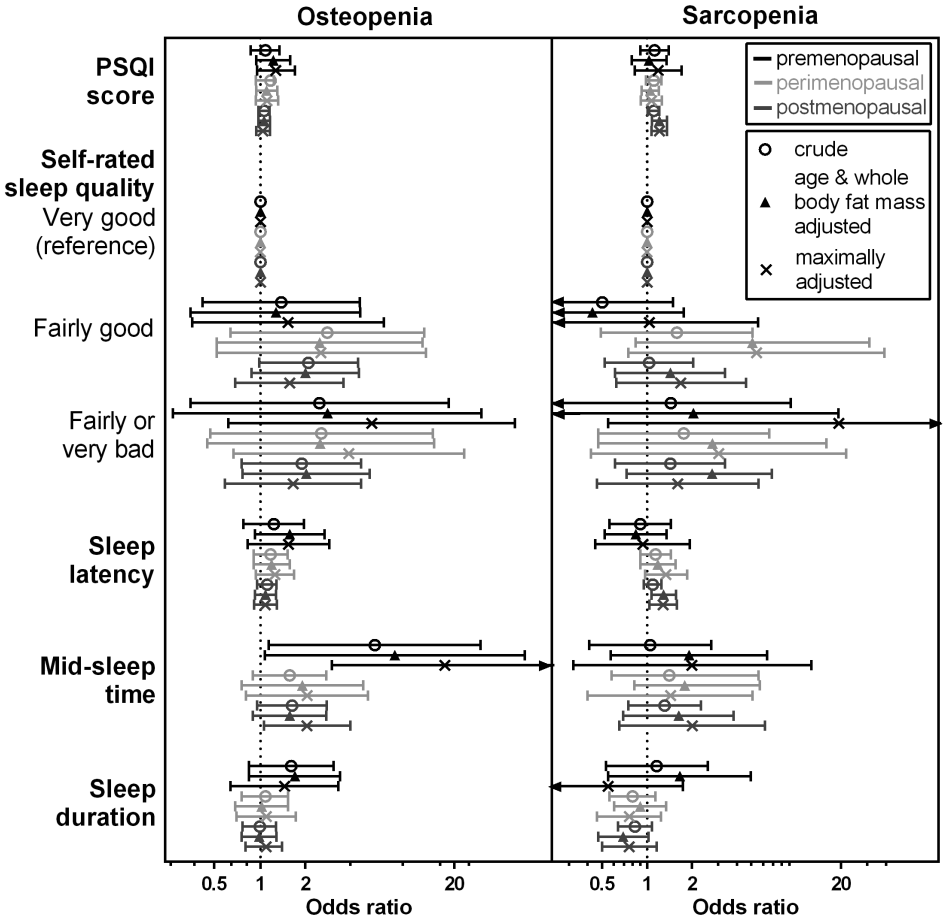


Fig. 2: OR (95% CI) for osteopenia and sarcopenia per unit change of sleep parameter stratified by sex. Crude, age & whole body fat mass adjusted, and maximally adjusted (age, whole body fat mass ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates, and menopause) models are shown. PSQI, Pittsburgh Sleep Quality Index. Results were based on analyses weighted towards the BMI distribution of the general population (*n* = 915).

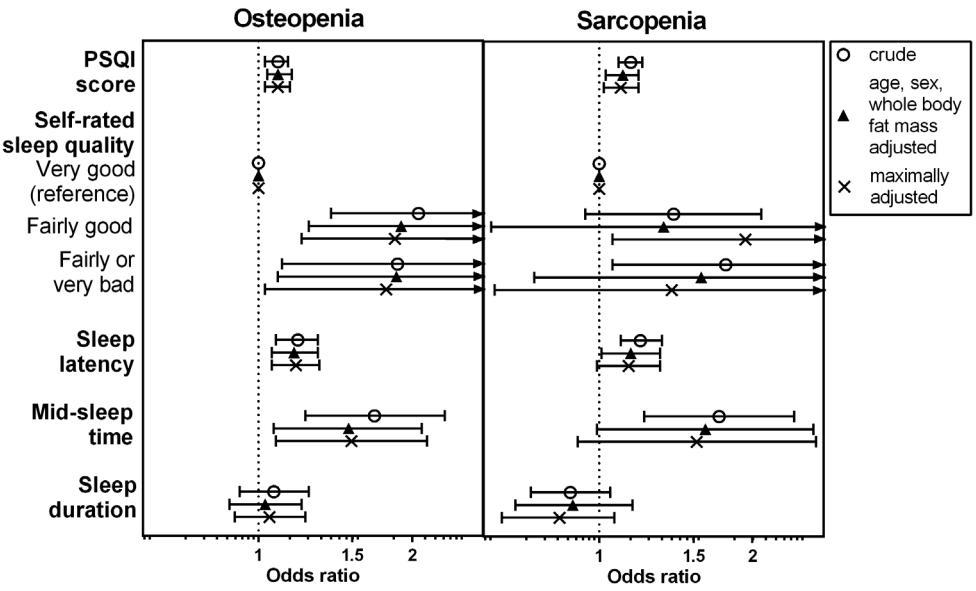
In analysis stratified by menopausal status (**Suppl. Fig. 1**), the association between mid-sleep time and osteopenia appeared to be strongest in premenopausal women, while PSQI score and sleep latency in postmenopausal women associated more with sarcopenia. Associations between sleep duration and osteopenia and sarcopenia were not observed in any of the menopausal groups.

Sleep parameters and risk of osteopenia adjusted for muscle mass and of sarcopenia adjusted for BMD

After additional adjustment of the models for osteopenia and sarcopenia for muscle mass and BMD, respectively (**Suppl. Fig. 2**). The effect sizes of the associations between sleep parameters on osteopenia were only marginally affected. *Vice versa*, when models for relating sleep parameters to sarcopenia were additionally adjusted for BMD of the spine or hip, results were also only slightly affected.



Suppl. Fig. 1: OR (95% CI) for osteopenia and sarcopenia per unit change of sleep parameter stratified per menopausal status. Crude; age & whole body fat mass adjusted; and maximally adjusted (age, whole body fat mass, ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates) models are shown. PSQI, Pittsburgh Sleep Quality Index. Results were based on analyses weighted towards the BMI distribution of the general population (*n* = 915).



Suppl. Fig. 2: OR (95% CI) for osteopenia and sarcopenia per unit change of sleep parameter. Maximally adjusted models are shown for reference (age, sex, whole body fat mass, ethnicity, education, alcohol intake, physical activity, vitamin D levels and season, usage of systemic corticosteroids and bisphosphonates (note that these models are also shown in **Fig. 1**)), and models were additionally adjusted for muscle mass, BMD spine, or BMD hip. PSQI, Pittsburgh Sleep Quality Index; BMD, bone mineral density. Results were based on analyses weighted towards the BMI distribution of the general population (*n* = 915).

Discussion

In this population-based study, we extensively examined associations of sleep duration, sleep timing and sleep quality with osteopenia and sarcopenia in middle-aged men and women. In our cohort, a decline in sleep quality measures was associated with an increased risk of osteopenia and sarcopenia. All measures of sleep quality (total PSQI score, self-reported sleep quality, and sleep latency) were consistently associated with musculoskeletal health, which adds to the validity of their relationship.

Individuals with a later sleep timing also exhibited a higher risk of osteopenia and a similar trend was observed for risk of sarcopenia. The mid-sleep time is a marker for chronotype, which constitutes the individual timing of behavior caused by the internal circadian phase relative to the light-dark cycle. The evening chronotype has been associated with higher levels of cortisol and of catecholamines,[2,6] which could in turn negatively affect the musculoskeletal system. These high levels of cortisol and catecholamines may be caused by the discrepancy between preferred and actual sleep timing that evening types often experience due to social constraints.[37] Chronic circadian misalignment has been related to metabolic derangements previously.[38,39] For instance, circadian disruption

induced by constant light worsened bone microstructure in young mice resembling age-related osteoporosis.[40] Alternatively, effects of sleep time on the musculoskeletal system may be partly explained by behavioral differences. Individuals exhibiting later sleep times display more unhealthy behaviors, such as smoking, increased alcohol usage,[41] less physical activity,[42] and later meal times.[5] However, associations between mid-sleep time and osteopenia/sarcopenia remained present after adjusting analyses for smoking and physical activity. Our results are consistent with studies that showed that postmenopausal women with bedtimes after midnight were at increased risk for osteoporosis[10] and that sarcopenia was more prevalent in middle-aged individuals with a later chronotype.[18]

Interestingly, the associations with sleep quality and timing with musculoskeletal health were stronger in women than in men, especially for mid-sleep time. Sex differences exist in the prevalence of osteopenia/sarcopenia[19,20] and in sleep parameters, with women sleeping longer[43] and spending more time in slow wave sleep than men.[43,44] The relationship between sleep and musculoskeletal health may also be influenced by sex-related differences in sex hormones, employment status, and social behavior. We observed some minor differences per menopausal status. The associations between sleep quality/timing and osteopenia were somewhat weaker in postmenopausal women than in premenopausal, perhaps because the lack of estrogen after the menopause exerts effects bone loss to such a strong degree[45] that bone loss is less dependent on sleep. On the other hand, the association between the PSQI score and sarcopenia was strongest in postmenopausal women, so sleep quality does associate with muscle mass while these women are losing muscle rapidly due to the loss of estrogen.[46]

We did not observe an association between sleep duration and osteopenia/sarcopenia. Based on experimental sleep deprivation studies, we expected that short sleep would elicit changes in mediators that could negatively affect musculoskeletal health, such as decreased IGF-1 levels and increased stress hormones.[2] However, a relatively low number of individuals (9%) slept less than the recommended 6 hrs per night[47] and more severe sleep deprivation may be necessary to induce such effects.[2] Moreover, our results are consistent with a study in 20 to 66 year old Americans that also did not observe an association between sleep duration and BMD.[7] Other studies have shown inconsistent results and were mostly performed in Asian subjects.[9-13] It has also been reported that longer sleeping postmenopausal women had a lower skeletal muscle mass,[8] while we did not observe this in postmenopausal women. Women in this study averagely slept 8.7 hrs while our postmenopausal women slept 7.0 hrs, which may account for the differences.

Associations between sleep quality and bone quality were similar in the spine and hip. Trabecular bone (typical in the spine) has a more rapid rate of age- and postmenopausal-related deterioration than cortical bone (typical in the hip) and is more responsive to cortisol,[48] suggesting that the associations between sleep and bone may be independent of these factors.

In line with previous studies[22,23,49] BMD was correlated with muscle mass in our study. However, we did not observe an association between osteopenia and sarcopenia and our additional statistical analyses suggest that the associations of sleep with osteopenia and sarcopenia were not mediated via muscle and BMD, respectively. This is somewhat surprising, since there is evidence that deterioration of the musculoskeletal system is characterized by bone loss and muscle wasting in parallel and that these processes share common determinants,[21,50] but may be explained by the relatively young age of our participants and the therefore low prevalence of osteopenia/sarcopenia. Therefore, the mechanisms linking sleep quality/timing with osteopenia and sarcopenia may be different in this cohort, which makes it even more striking that sleep quality/timing associate with both osteopenia and sarcopenia. Exhibiting both conditions will greatly increase the risk for fractures and frailty.[22,23]

There are several pathophysiological mechanisms that may explain the relation between sleep and musculoskeletal health. One mechanism is *via* endocrine factors known to be affected by sleep and influencing musculoskeletal health. A decline in sleep quality may prevent individuals from reaching the deepest sleep stages,[3] which are thought to be the most "restorative" and these are the times at which most GH is secreted.[51] GH exerts effects on the musculoskeletal system directly and through mediating insulin-like growth factor I (IGF-1) levels.[52] GH and IGF-1 levels progressively decline in aging and are important contributors to age-associated osteopenia and sarcopenia.[52,53] In addition to GH, studies that experimentally fragmented sleep reported higher cortisol levels and a higher sympathovagal balance,[2-4] which can exert detrimental effects on the musculoskeletal system directly and by inhibiting GH and IGF-1.[5,52] Additionally, observational studies report a positive association IGF-1 levels and amount spent in deep sleep.[54,55]

We observed that sleep quality measures associated only weakly with BMD in the spine and hip, implying that there may be a cut-off point of more progressed stages of bone and muscle loss that are associated with insufficient sleep.

A weakness of this study is that it is observational and cross-sectional, so we cannot exclude residual confounding and reverse causation, precluding causal inference. Individuals with osteopenia and sarcopenia could suffer from inadequate sleep. Furthermore, likely due to their young age,[56] only 5% of participants in our cohort had osteoporosis, which prevented us to reliably determine the associations of sleep parameters with osteoporosis. Nevertheless, the majority of individuals with osteopenia will likely develop osteoporosis in the next 10 years.[57] Although we assessed sleep quality with an extensive questionnaire and previous studies linking sleep to bone health used one single question,[14-16] the use of sleep diaries and actigraphy may be even more reliable methods to assess sleep. Furthermore, the usage of chronotype questionnaires may more reliably assess preference for sleep timing than mid-sleep time alone.[58]

Strengths of the study were the reliable assessment of BMD and muscle mass by DXA, in contrast to quantitative ultrasound[13,14] or bioimpedance analysis,[8] in a large study population, including extensive measurement of potential confounding factors at baseline, including demographic factors, body composition, alcohol intake, physical activity, vitamin D levels and medication usage. Furthermore, the inclusion of assessing both osteopenia and sarcopenia allowed us to determine their independent and combined associations with sleep parameters and to identify potential interactions.

Taken together, decreased sleep quality and a later sleep timing contribute to increased risk of osteopenia and sarcopenia in the middle-aged. Future studies should prospectively examine the associations of sleep with musculoskeletal health and fracture risk. It has been demonstrated that sleep quality can be improved by the implementation of personalized sleep hygiene strategies,[59] offering a pathway for sleep interventional studies to study the causative effect of sleep on musculoskeletal health.

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Appendix NEO Study Group

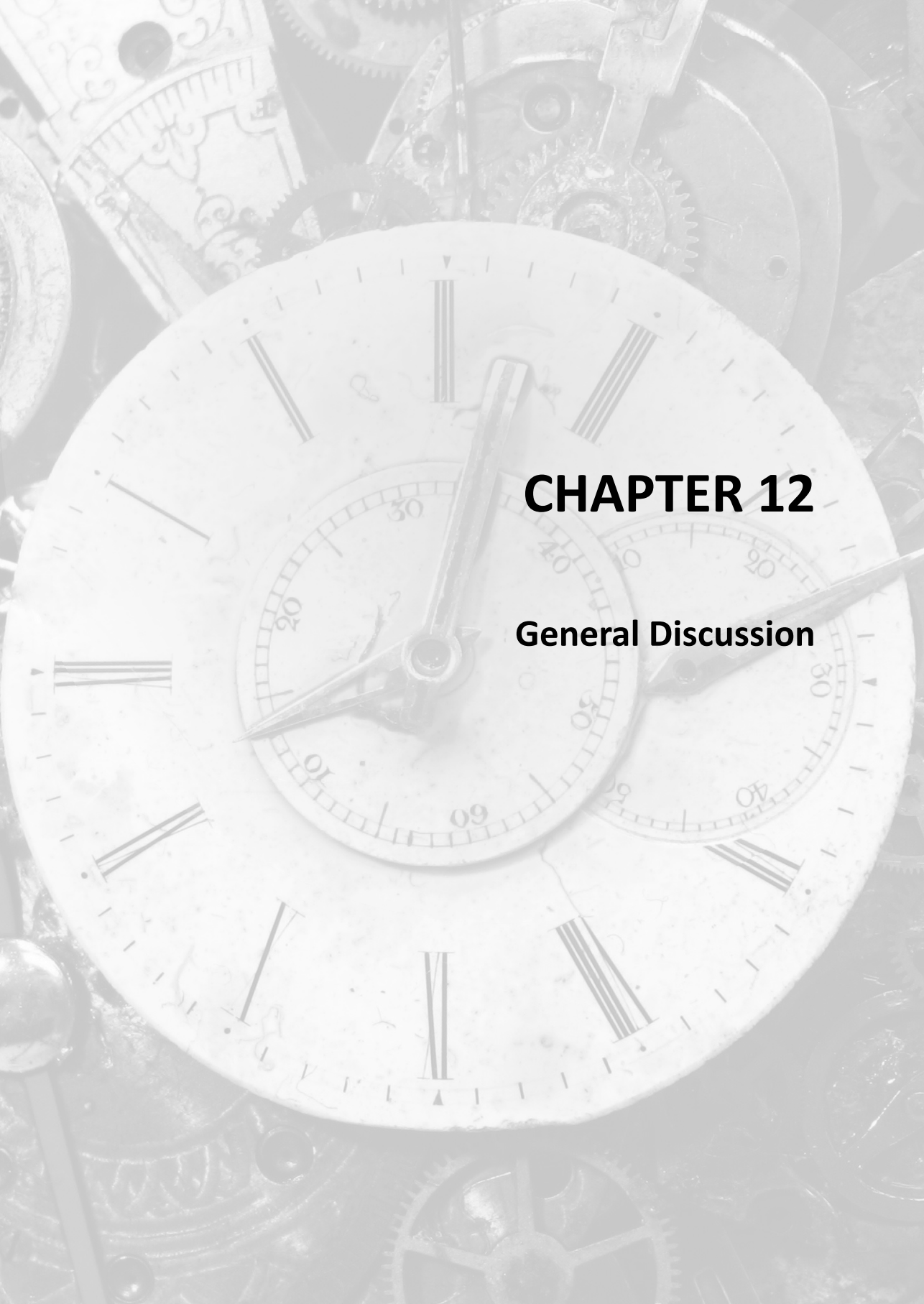
Frits R. Rosendaal, Renée de Mutsert, Ton J. Rabelink, J. Wouter Jukema, Albert de Roos, Ko Willems van Dijk, Saskia le Cessie, Pieter S. Hiemstra, Margreet Kloppenburg, Anton J.M. de Craen, Eelco J.P. de Koning, Pieter H. Reitsma, Aiko P.J. de Vries, Hildo J. Lamb, Ingrid M. Jazet, Olaf M. Dekkers, Nienke R. Biermasz, Christa M. Cobbaert (Leiden University and Medical Center, Leiden, the Netherlands), Martin den Heijer (VU Medical Center, Amsterdam, The Netherlands)

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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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Summary
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SUMMARY

To be optimally adjusted to life on earth, nearly all bodily processes display a circadian rhythm. These rhythms of about 24 hours (*circa* = around, *dies* = a day) result from a molecular clockwork and are orchestrated by the suprachiasmatic nuclei (SCN) in the anterior hypothalamus. The SCN are endogenous pacemakers that are synchronized to the environment mainly by light. Light reaches the SCN via the retinohypothalamic tract (RHT) and can shift the phase of the endogenous circadian rhythm depending on the time of exposure. The SCN contain about 20,000 neurons which are coupled and together produce a robust multi-unit output rhythm. The duration of day length is reflected by a broadening or shortening of the waveform of the SCN output rhythm which is achieved by altering the phase relationship amongst neurons. The SCN transfer their output to various pathways and influence the timing of many physiological processes and behavior, for example sleep. Sleep timing is additionally influenced by sleep pressure, a homeostatic drive for sleep, which increases during wakefulness and decreases during sleep mainly because of rising and declining levels of adenosine, respectively. Sleep normally consists of cycles of sleep stages that can be roughly divided in rapid eye movement (REM) and nonrapid eye movement (NREM) sleep. The individual preference for sleep timing is referred to as “chronotype” and generally distinguishes morning and evening types.

In modern society, irregular timing of behavior and light exposure is progressively common, which can be disruptive for circadian rhythms. Sleep deprivation is also frequently observed. Insufficient sleep and disrupted circadian rhythms have been related to a spectrum of health consequences, which are discussed extensively in **Chapter 2** and **3**. In order to fully comprehend these associations, an understanding of the basic functioning of sleep and the circadian system is necessary.

In **Chapter 4**, we studied the electrophysiological and behavioral characteristics of mice lacking the neurotransmitter vasoactive intestinal peptide (VIP), which is normally present in the SCN. We found that VIP KO mice behave normally and display normal neuronal SCN rhythms *in vivo* when they are in a light-dark environment. However, when transferred to constant darkness, VIP KO differed considerably from wildtype mice. For instance, while wildtype mice had encoded the preceding day length, VIP KO mice failed to adapt to short and long days. This indicates that VIP-mediated intercellular coupling is needed for the neuronal reorganisation of the SCN network necessary for seasonal adaptation. It also shows that the circadian system lacking VIP can be “rescued” by receiving proper photic input, possibly regulated via the RHT or through behavioral feedback effects.

In **Chapter 5**, effects of the widely used psychostimulant caffeine, an antagonist of the adenosine receptor, on sleep and the circadian system were examined in mice. The light response of the SCN was attenuated after a 6hr sleep deprivation, and subsequent



intraperitoneal application of caffeine restored the response to light. This implicates that adenosine is involved in modulating the light sensitivity of the circadian system. Further evidence for this was provided by the observation that exposure to constant light lengthened the circadian period in mice and that this effect was enhanced by chronic caffeine treatment. Importantly, in constant dark, caffeine treatment did not significantly alter the period duration. Extrapolating these results to humans, light exposure in combination with caffeine consumption in the morning would produce a greater phase advance of the circadian rhythm than light exposure alone. Therefore, these results may provide a new pathway for improving circadian synchronisation to the environment, which may be particularly useful for extreme chronotypes and in jet lag.

In **Chapter 6**, we investigated the relationship between behavioral activity and the circadian system in freely moving mice. Spontaneous behavioral activity was associated with a concurrent suppression of SCN neuronal activity. More intense behavioral activity related to a deeper suppression. Since mice are nocturnal animals and the SCN neuronal activity is usually low at night, behavioral activity at night and rest during the day increased the amplitude of the SCN overall circadian rhythm. Since humans are day active and human SCN activity also peaks during the day, it would be interesting to repeat these experiments in diurnal animals and investigate whether active behavior at the proper time in the circadian cycle also boosts the rhythm. A high amplitude circadian SCN output rhythm is beneficial for health as it helps the body adjust to environmental differences between day and night.

In **Chapter 7**, we examined the metabolic effect of a complete loss of the SCN-driven circadian rhythmicity by lesioning the SCN in mice. Lesioned mice lost rhythms in behavioral activity, oxygen consumption, and food intake. While total food intake and behavioral activity was similar in lesioned mice and sham-lesioned mice 8 weeks after the lesion, lesioned mice were heavier, had a higher fat mass, displayed severe hepatic insulin resistance as measured by hyperinsulinemic-euglycemic clamp analysis. This shows that SCN is crucial in maintaining energy balance and hepatic insulin sensitivity.

In **Chapter 8**, we examined the effect of attenuated circadian rhythms on several health parameters in mice. Mice were exposed to constant light for 24 weeks, which dampened circadian rhythms in behavior and in the SCN. Over time, constant light-exposed mice became heavier, had a worse performance on a functional muscle test regime and displayed osteoporosis-like changes in the microstructure of their bones, with declining trabecular bone volume and structure. Upon restoration of a robust light-dark cycle, circadian rhythms recovered and muscle strength and bone structure normalized. Constant light also induced a more pro-inflammatory and responsive state of the immune system after 2 and 8 weeks, which normalized after longer constant light exposure. Taken together, these findings demonstrate that a reduced circadian rhythm induces reversible detrimental effects on health and that a robustly cyclic environment would be beneficial for health.

In **Chapter 9**, the relationship between chronotype and cardiovascular predictors is examined in short sleeping obese individuals. With the Horne and Ostberg Morningness-Eveningness questionnaire, 119 individuals were divided into morning or evening types. Eveningness was associated with eating later in the day, having less eating occasions and eating larger portion sizes, which is considered a more unhealthy eating pattern. Furthermore, eveningness associated with a higher BMI, a higher resting health rate, higher urinary epinephrine and plasma ACTH levels. Thus, in obese short sleeping individuals, a population that is already at increased risk for cardiovascular disease, eveningness negatively affects cardiovascular predictors further and awareness should be raised for sleep timing in this population.

In **Chapter 10,** we investigated the effect of sleep extension on neurocognitive function in the same population of short sleeping obese individuals. At baseline, a large proportion of these individuals had an impaired global deficit score. Sleep was improved in a nonpharmalogical manner for about one year and individuals visited the clinic regularly. At the final evaluation, subjective sleep quality and sleep duration improved. Global cognitive function also improved, especially in the attention domain. The improvement in cognitive function was associated with the amelioration in sleep parameters. In conclusion, sleep extension could be a pathway to enhance neurocognitive function in this vulnerable population.

In **Chapter 11**, we associated sleep with musculoskeletal parameters in the cohort of the Netherlands Epidemiology of Obesity Study (NEO). A lower subjective sleep quality and a later sleep timing associated with an increased risk for osteopenia (low bone mass) and sarcopenia (low muscle mass; association sleep timing - sarcopenia was borderline significant). Thus, subjectively sleeping worse and sleeping later associate with declined musculoskeletal health and puts individuals at an increased risk for fractures and frailty.

Taken together, studies presented in this thesis provide proof for associations between altered circadian rhythms / insufficient sleep and unfavourable health outcomes, ranging from obesity, insulin resistance, and increased cardiovascular risk factors, to deteriorations in musculoskeletal parameters, the immune system, and neurocognitive function. Studies also provide insight in potential valuable strategies to improve health by ameliorating sleep and enhancing circadian amplitude by caffeine, by appropriately timed behavioural activity and rest, and by suitable exposure to light at the proper phase of te light dark cycle.



NEDERLANDSE SAMENVATTING

Bijna alle lichaamsprocessen vertonen een circadiaan ritme, waardoor we optimaal aangepast zijn aan het leven op aarde. Deze ritmes van ongeveer 24 uur (*circa* = rondom, *dies* = een dag) worden aangedreven door een moleculair klokwerk en worden aangestuurd door de suprachiasmatische kernen (SCN) in de anteriore hypothalamus. De SCN zijn endogene pacemakers die vooral door licht met de omgeving worden gesynchroniseerd. Licht bereikt de SCN via de tractus retinohypothalamicus (RHT) en kan de fase van het endogene circadiane ritme verschuiven afhankelijk van het tijdstip van blootstelling. De SCN bevatten ongeveer 20.000 neuronen die gekoppeld zijn en samen een robuust multi-unit outputsignaal produceren. De duur van de daglengte wordt gereflecteerd door een verbreding of versmalling van de golfvorm van het outputritme van de SCN. De SCN sturen hun output naar meerdere systemen en beïnvloeden zo de timing van verschillende fysiologische processen en van gedrag, bijvoorbeeld van slaap. De timing van slaap wordt ook beïnvloed door slaapdruk, de homeostatische regulatie van slaap, die toeneemt tijdens wakker zijn en afneemt tijdens slaap, door toedoen van respectievelijk toenemende en afnemende niveaus van adenosine. Slaap bestaat normaliter uit cycli van slaapstadia die ruwweg kunnen worden verdeeld in *rapid eye movement* (REM) en *nonrapid eye movement* (NREM) slaap. De individuele voorkeur voor de timing van slaap wordt ook wel "*chronotype*" genoemd waarbij een onderscheid kan worden gemaakt tussen ochtend- en avondmensen.

In de moderne maatschappij komt onregelmatige timing van gedrag en blootstelling aan licht steeds vaker voor. Ook slaapdeprivatie komt vaker voor. Insufficiënte slaap en verstoorde circadiane ritmiek zijn gerelateerd aan een spectrum van gezondheidseffecten, die besproken worden in **Hoofdstuk 2** en **3**. Om deze associaties vollediger te kunnen begrijpen is ook begrip van de basale functie van slaap en het circadiane systeem noodzakelijk.

In **Hoofdstuk 4** hebben we de elektrofysiologische en gedragsmatige karakteristieken bestudeerd van muizen die de neurotransmitter *vasoactive intestinal peptide* (VIP) missen, die normaliter onder andere in de SCN aanwezig is. In licht-donker omstandigheden vertoonden deze VIP KO muizen normaal gedrag en een normale neuronale SCN ritmiek *in vivo*. Wanneer zij echter in een constant donkere omgeving werden geplaatst verschillen zij sterk van wildtype muizen. Wildtype muizen sloegen bijvoorbeeld de voorafgaande daglengte op in de SCN, maar VIP KO muizen niet. Dit geeft aan dat VIP-gemedieerde intercellulaire koppeling nodig is voor de neuronale reorganisatie van het SCN netwerk die optreedt bij seizoenale adaptatie. Het demonstreert tevens dat het circadiane systeem zonder VIP door lichtinput toch "gered" kan worden, mogelijk gereguleerd door de RHT of door gedragsmatige feedbackmechanismen.

In **Hoofdstuk 5** worden in muizen de effecten op slaap en op het circadiane systeem onderzocht van de wereldwijd gebruikte psychostimulant cafeïne, een antagonist van de



adenosinereceptor. De lichtrespons van de SCN was verminderd na een slaapdeprivatie van 6 uur en de daaropvolgende intraperitoneale injectie van cafeïne herstelde de lichtrespons. Dit impliceert dat adenosine betrokken is bij het moduleren van de lichtgevoeligheid van het circadiane systeem. Verder bewijs hiervoor werd geleverd door de observatie dat blootstelling aan constant licht de circadiane periode verlengde en dat dit effect versterkt werd door chronische behandeling met cafeïne. Nota bene, in constant donker had behandeling met cafeïne geen significant effect op de periode. Wanneer we deze resultaten extrapoleren naar mensen, zou 's ochtends lichtblootstelling in combinatie met cafeïneconsumptie een grotere faseversnelling van het circadiane systeem bewerkstelligen dan alleen lichtblootstelling. Daarom zijn deze resultaten mogelijk een nieuwe manier om circadiane synchronisatie met de omgeving te versnellen, wat vooral nuttig zou kunnen zijn voor extreme chronotypen en bij jetlag.

In **Hoofdstuk 6** onderzochten we de relatie tussen gedragsactiviteit en het circadiane systeem in vrij bewegende muizen. Spontane gedragsactiviteit was geassocieerd met een gelijktijdige onderdrukking van de neuronale activiteit van de SCN. Intensiever gedrag was gerelateerd aan een diepere onderdrukking. Omdat muizen nachtdieren zijn en de neuronale activiteit van de SCN 's nachts normaliter laag is, zorgen nachtelijke gedragsactiviteit en rust gedurende de dag dus voor een verhoging van de amplitude van het circadiane SCN ritme. Mensen zijn dag-actief en de SCN activiteit van mensen piekt ook overdag, dus het zou interessant zijn om te onderzoeken of actief gedrag op de juiste tijd in de circadiane cyclus bij hen ook het neuronale ritme versterkt. Een SCN outputritme van hoge amplitude komt de gezondheid ten goede omdat dit het lichaam helpt aanpassen aan de omgevingsverschillen tussen dag en nacht.

In **Hoofdstuk 7** onderzochten we het metabole effect van een compleet verlies van de centrale aansturing van circadiane ritmiek door de SCN van muizen te laederen. Geledeerde muizen verloren ritmiek in gedragsactiviteit, zuurtsofconsumptie en voedselinname. Terwijl de totale voedselinname en gedragsactiviteit van gelaedeerde muizen vergelijkbaar was met die van muizen met een sham lesie, waren gelaedeerde muizen zwaarder, hadden zij een hogere vetmassa en hadden zij ernstige insulineresistentie in de lever (gemeten met hyperinsulinemische-euglycemische clampanalyse). Dit toont aan dat de SCN cruciaal is bij het onderhouden van de energiebalans en de insulinegevoeligheid in de lever.

In **Hoofdstuk 8** werd het effect van verminderde circadiane ritmiek op verscheidene gezondheidsparameters in muizen onderzocht. Muizen werden gedurende 24 weken blootgesteld aan constant licht, waardoor de circadiane ritmiek in hun gedrag en in de SCN sterk werden verminderd. Muizen die aan constant licht waren blootgesteld werden zwaarder, hadden een slechter resultaat op functionele spiertesten en hadden osteoporoseachtige veranderingen in de microstructuur van hun botten, met verminderde trabeculaire volumina en verslechterde structuur. Nadat het licht-donker schema was hersteld, herstelde

de circadiane ritmiek zich en normaliseerden de spierkracht en botstructuur. Constant licht induceerde ook een meer pro-inflammatoire staat van het immuunsysteem na twee en acht weken, die weer normaliseerde na een langere blootstelling. Deze bevindingen demonstreren dat verminderde circadiane ritmiek omkeerbare negatieve gezondheidseffecten veroorzakt en dat een robuust ritmische omgeving gezondheidswinst op zou kunnen leveren.

In **Hoofdstuk 9** onderzochten we de relatie tussen chronotype en cardiovasculaire voorspellers in kort slapende obese personen. Met behulp van de Horne en Ostberg Morningness-Eveningness vragenlijst werden 119 personen verdeeld in ochtend- of avondmensen. Personen die lager scoorden op de vragenlijst (meer richting avondmens) hadden latere eetmomenten, minder eetmomenten en aten grotere porties – dit is een ongezonder eetpatroon. De avondmensen hadden ook een hogere BMI, een hogere rusthartslag en hogere urinaire epinefrine- en plasma ACTH niveaus. In obese kort slapende personen, een populatie die al een hoger risico heeft op cardiovasculaire ziekten, lijken de avondmensen dus een nog hoger risico op ziekte te lopen. Daarom zou er in deze populatie meer aandacht moeten zijn voor sleep *timing*.

In **Hoofdstuk 10** werd het effect van verlenging van slaap op neurocognitieve functies onderzocht in dezelfde populatie van kort slapende obese personen. Aan het begin van de studie had een groot deel van deze personen een verminderde globale cognitieve functie. Slaap werd gedurende ongeveer één jaar door middel van individuele coaching op non farmacologische wijze verbeterd. Bij de laatste evaluatie waren de subjectieve slaapkwaliteit en de slaapduur verbeterd. De globale cognitieve functie was ook verbeterd, met name in het aandachtsdomein. Deze verbetering in cognitie was geassocieerd met de verbetering van slaapparameters. Concluderend zou verlenging van slaap dus een manier kunnen zijn om neurocognitieve functies te verbeteren in deze kwetsbare populatie.

In **Hoofdstuk 11** associeerden we slaap met musculoskeletale parameters in het cohort van de Nederlandse Epidemiologie van Obesitas studie (NEO). Een lagere subjectieve slaapkwaliteit en een latere *timing* van slaap waren geassocieerd met een verhoogd risico op osteopenie (een lage botmassa) en sarcopenie (lage spiermassa; de associatie sleep *timing* – sarcopenie was borderline significant). Subjectief slechter slapen en later slapen zijn dus geassocieerd met verslechterde musculoskeletale gezondheid en verhogen het individuele risico op fracturen en kwetsbaarheid.

Deze studies leveren het bewijs dat er associaties bestaan tussen veranderde circadiane ritmiek / insufficiënte slaap en ongewenste gezondheidsuitkomsten, van obesitas, insulineresistentie en verhoogde cardiovasculaire risicofactoren, tot verslechterde musculoskeletale parameters, veranderingen in het immuunsysteem en in neurocognitieve functie. De studies geven ook inzicht in potentieel waardevolle strategieën om gezondheid te versterken door slaap en circadiane amplitude te verbeteren door bijvoorbeeld cafeïne en goed getimed gedrag en lichtblootstelling.



CURRICULUM VITAE

Eliane Lucassen werd geboren op 3 oktober 1986 in Leiden. In 2004 behaalde zij *cum laude* haar VWO diploma aan het Bonaventura College in Leiden en startte zij aan de studie Geneeskunde aan de Universiteit Leiden. In 2005 nam zij deel aan een uitwisseling met het Karolinska Institutet in Stockholm. Het jaar daarop startte zij via het "excellente studententraject" bij het lab Neurofysiologie in het LUMC onder dagelijkse supervisie van Dr. Thijs Houben. In 2007 startte zij met de pre-master Biomedische Wetenschappen. Eliane was actief in het studentenleven in verscheidene commissies en zij is voorzitter geweest van de internationale studentenvereniging AEGEE. Zij deed de master "Health" van Biomedische Wetenschappen met stages bij het lab Neurofysiologie in samenwerking met de afdeling Endocrinologie. Daarna volgde zij haar co-schappen en haar semi arts stage deed ze bij de afdeling interne geneeskunde in het Bronovo. In 2011 en 2012 studeerde ze *cum laude* af voor respectievelijk Geneeskunde en Biomedische wetenschappen.

Na haar afstuderen reisde ze eind 2011 af naar Amerika met financiële steun van Fulbright, het VSBfonds en LUF. Zij deed gedurende een jaar onderzoek bij de National Institutes of Health (NIH) in de *Sleep Extension Study Group* naar de relatie tussen slaap en endocrinologische factoren in obese proefpersonen. Eind 2012 startte zij haar officiële promotieonderzoek via het 2-jarige MD PhD traject in het LUMC onder leiding van Prof. dr. J.H. Meijer en zette zij een interdisciplinair project op naar de gezondheidseffecten van constante blootstelling aan licht. Daarnaast onderzocht zij de relatie tussen slaap en musculoskeletale parameters in de *Nederlandse Epidemiologie van Obesitas* studie. Zij onderbrak deze periode om in 2014 gedurende enkele maanden als arts-assistent (ANIOS) interne geneeskunde te werken in het Medisch Centrum Haaglanden.

Momenteel werkt Eliane als arts-assistent in opleiding tot specialist (AIOS) in het Medisch Centrum Haaglanden en Bronovo-Nebo. Zij specialiseert zich in de interne geneeskunde.



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Zonder de hulp van een heel aantal mensen was dit boekje nooit tot stand gekomen en daarom is dit misschien wel het moeilijkste en meest herschreven deel van het hele boekje. Lang geleden, als jonge studente, trad ik vol enthousiasme in de wetenschap onder begeleiding van Joke en Thijs, die me met veel aandacht mijn eerste introductie in de wetenschap hebben gegeven. Met de jaren heb ik veel mensen op ons lab zien komen en gaan. Robert, Tirsa, Maarten, Marcel, Danielle, en andere tijdelijke bewoners van T5-11, ik ben dankbaar dat ik ben blijven hangen in de "studentenkamer" en daardoor dagelijks door jullie omringd was. Yuri, ik ben blij dat we samen deze kamer hebben geïnfiltreerd. Het begeleiden van mijn topteam "eigen" studenten, Suzanne, Jasper, Robbert, Leonie en Sanne, was een drijfveer en jullie inzet was inspirerend. Hans, Heleen, Peter, Mayke, Jos, Tom, Stephan, Karim en Ashna, bedankt voor al jullie hulp en de mooie tijden samen. Maaike, vrolijke druif, ik vond het fijn om met jou samen te werken. Renate, bedankt voor je eerlijke meningen en de gedachtenwisselingen over belangrijke dingen van het leven en van de wetenschap.

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LIST OF PUBLICATIONS

ORIGINAL RESEARCH ARTICLES

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