Cardiovascular control by the biological clock

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CHAPTER 1

The ins and outs of the clock

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A. THE CLOCKWORK

From the beginning, life on earth has been exposed to sunlight in a daily rhythm as determined by the earth's rotation. Sunlight is the main energy source for all organisms, directly, by photosynthesis, or indirectly, via the food chain. Furthermore, sunlight heats the environment and allows photic vision. The rhythmic appearance of sunlight thus allows and dictates the life of an organism. Through millions of years, evolutionary pres-
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Sure has favored those species that are able to anticipate these rhythmic environmental changes. In mammals, this has led to the development of a specialized brain region dedicated to this function, the 'biological clock'.

During normal working days with regular sleep-wake cycles, we are quite unaware of the impact of our biological clock on body and mind. Even the sleep-wake cycle - the day-night rhythm we are most aware of - we do not perceive as a product of our endogenous clock. We tend to think that the build-up (during the day) and replenishment (during the night) of sleep pressure determines our sleep-wake cycle. Only when our sleep-wake is no longer in synchrony with the internal rhythm of our biological clock, the importance of the biological clock becomes apparent. This may be due to external shifts in the sleep-wake cycle relative to the endogenous rhythm of the biological clock (e.g. shift-work and jet lag) or to disturbances of clock function itself (e.g. Alzheimer disease [190] and delayed sleep phase syndrome [331]). In shift-work, for example, the rapid shifts in desired sleep-wake pattern by far exceed the biological flexibility of the biological clock, tailored to 24-hour rhythmicity. Obvious and immediate problems are insomnia and lowered alertness, of which the latter may result in fatal accidents and catastrophes. The risk of traffic and industrial accidents peaks during the second half of the night, when our core body temperature (BT) reaches its daily minimum and our fatigue its maximum [262]. Long-term, and thus less obvious, problems of shift-work include increased risk of cardiovascular disease, diabetes, and gastritis [167, 251]. Already, these few examples provide us a first glance at the importance of the biological clock in our daily lives.

Master clock

The mammalian biological clock generates the circadian (=approximately 24-h) rhythms in physiology and behavior. It is located in the suprachiasmatic nucleus (SCN) in the anterior hypothalamus. The SCN is the master oscillator of the organism, because it is in itself sufficient for the generation of a circadian rhythm, and because it is required for the circadian rhythms of the body. An isolated SCN in vivo [138] and in vitro [34, 106, 115, 302], and even single isolated SCN-neurons in vitro [363], retain their circadian rhythm. Lesioning the SCN eliminates the circadian rhythms of the animal [231, 316]. Furthermore, transplantation of an SCN to an arrhythmic, SCN-lesioned (SCNx), animal restores the circadian rhythm with properties (i.e. period length) of the donor, not the recipient [263]. Together, these results demonstrate that the SCN is the master clock that allows the organism to anticipate oncoming circadian changes and to synchronize its organs to the light-dark cycle.

Molecular clockwork

The current idea is that the mammalian clockwork of the SCN is based upon a cellular
transcriptional/translational feedback-loop with a period of about 24 h. The basic mechanism is the suppression by clock proteins of their own transcription. It is proposed that the clock proteins PERIOD (PER1 and PER2) and CRYPTOCHROMES (CRY1 and CRY2) form the heart of the clockwork [146,341]. The expression of these proteins varies with circadian phase, and follows their mRNA cycles. The BMAL-CLOCK complex stimulates the transcription of the PER and CRY genes, and their protein products suppress the effects of this complex. How the molecular clock could determine the circadian rhythm of the organism will be discussed later (see “CLOCK OUTPUT”, page 22).

**Neuronal clockwork**

By virtue of the aforementioned autoregulatory feedback loop, individual SCN neurons have the ability to maintain a circadian rhythm. However, such a cellular clock is far from precise, with individual neuronal period lengths varying from 20 to 28 hours, and a standard deviation (SD) of about 1.2 hours [130,134,363]. However, within the SCN, the individual neurons form a neuronal network which increases the circadian precision, and the SD is reduced to about 0.4 hours [130]. The even further enhanced precision of the circadian output of the SCN *in vivo*, within the range of 24.0-24.8 hours (SD 0.2 hours), indicates that the reciprocal neuronal communication of the SCN with other hypothalamic nuclei add to the precision of the circadian rhythm generated by the SCN [130,134]. Possible neurotransmitters involved in communication within the SCN are discussed in the next part.

**Neurotransmitters of the SCN**

Although the SCN is a small nucleus (not even 1 mm³), it contains over 10,000 neurons that are packed tightly together, with a heterogeneous neuron population containing multiple neurotransmitters. In the dorsomedial part of the SCN, vasopressin (VP) positive neurons prevail. In the ventrolateral part, the part that receives photic input from the retina, neurons synthesize vasoactive intestinal polypeptide (VIP), gastrin-releasing peptide (GRP) and/or peptide histidine isoleucine (PHI). In between these two cell populations a smaller, distinct population of somatostatin (SOM)-positive neurons is found. γ-Amino-butyric acid (GABA) is present throughout the SCN and colocalizes with above-mentioned neurotransmitters [273,274]. Besides these well-described neurotransmitter populations, many more neurotransmitters have been reported to be present, e.g. calbindin, thyrotrophin-releasing hormone, corticotrophin-releasing hormone, angiotensin II, enkephalin, substance P, dynorphin, cholecystokinin, calcitonin gene-related peptide, and galanin [342].

Interestingly, of all these neurotransmitters, only for VP a clear circadian rhythm in production and release has been demonstrated. VP has an endogenous circadian rhythmic expression in the SCN, thus not only under light-dark (LD) conditions, but also
under constant darkness (DD). Although VP is the most studied neurotransmitter in the SCN, little is known about its function. Often it is concluded that VP is not required for the expression of circadian rhythmicity of the animal, as suggested by the circadian rhythms present in the VP-deficient Brattleboro rat. However, one should be cautious with interpretation of studies in (natural) knockout (KO) animals. In KO-animals, biological adaptation and flexibility during development could mask the importance of a gene and its product (i.e. VP) in a system (i.e. circadian system). VP neurons are part of the efferent system of the SCN. VP from the SCN, for example, plays a crucial role in the circadian regulation of corticosterone secretion and reproduction in the rat [46, 151, 158, 247].

VIP-positive neurons are located in the ventrolateral part of the SCN and receive light input from the retinohypothalamic tract (RHT) and the intergeniculate leaflet of the thalamus (IGL). There is no endogenous rhythm in VIP expression or in its receptor VPAC2 in the SCN [305]. However, during LD conditions, there is a rhythm in VIP expression with low levels during the day and two peaks just after lights off and just before lights on [305]. VIP application phase-shifts VP neurons in the dorsomedial SCN similar to phase-shifts induced by light exposure [357]. The sequential appearance of fos expression, first in the ventrolateral-VIP part and then in the dorsomedial-VP part, indeed [272] suggests that VIP cells communicate SCN-input to the rest of the SCN.

GABA is present throughout the SCN and is colocalized with most of the abovementioned neurotransmitters. There is indirect evidence that GABA release from the SCN fluctuates in a rhythmic way, both under LD and DD conditions, with peak levels during the (subjective) day period [157]. The widespread presence of GABAergic synapses between individual SCN neurons suggests a critical role of GABA also within the SCN [319]. There are however conflicting results on this function of GABA within the SCN. Wagner and coworkers reported that GABA enhances the circadian rhythm amplitude by exciting other SCN neurons during the day and inhibiting them during the night period [354]. However, Gribkoff and coworkers report that GABA is inhibitory during the daytime [114]. The exact function of GABA within the SCN requires further research. Functions of GABA in the efferent system of the SCN have, however, been clearly demonstrated (see “CLOCK OUTPUT”, page 22).

Comparing SCN of nocturnal and diurnal mammals, including man

Both in night-active (nocturnal) and day-active (diurnal) mammals, electrophysiological measurements of SCN neurons show that most SCN neurons are active during the day-period and inactive during the night-period [115, 215, 287, 302]. Also, the circadian rhythm in metabolic activity is similar in diurnal and nocturnal animals, with higher levels at mid-day [296]. In humans, the critical role of the SCN can obviously not be experimentally tested by means of lesion studies, although two unique case reports sug-
suggest that, also in humans, lesions of the SCN, e.g. due to surgery of a tumor, lead to disruption of circadian rhythmicity [64, 294]. However, all anatomical and physiological similarities of the circadian system between humans and other mammals provide convincing evidence for the critical role of the SCN in humans. Firstly, the location of the human SCN is similar to that in other mammals, residing as it does in the anterior hypothalamus, bilaterally next to the third ventricle, and on top of the optic chiasm. Secondly, the projections from the retina to the SCN and from the SCN to other hypothalamic target areas in humans are very similar to those in rats [70, 71]. Light information from the retina reaches the ventral part of the SCN via the RHT similarly in humans and rodents [71]. In both rats and humans, the SCN projects to the PVN, the DMH and the PVT. Thirdly, both human and rat SCN use the neurotransmitters VP, VIP and GABA. In rat and human, VP-positive neurons dominate the dorsal part and VIP-positive neurons are restricted to the ventral part, while GABA is dispersed over the SCN [70]. The human SCN additionally contains neurotensin as an important neurotransmitter. Also, the day-night rhythm in VP content of the human SCN has been demonstrated by post mortem study, showing a peak in the early morning [133]. Finally, the circadian system of humans and other animals is similar in functional respects. Just like other mammals, human endogenous circadian rhythms can be phase-shifted by light and by the hormone melatonin, the most important day and night signals for the SCN, respectively [29, 68, 171, 186, 193, 206, 217, 282]. The phase response curves light [226, 277, 339] and melatonin [185, 300, 376] are qualitatively the same as in other animals, in which it has been demonstrated that the SCN is necessary for this function [206, 277]. In humans, too, melatonin secretion is immediately inhibited by light [30, 38, 378], just as in rats, in which the critical role for the SCN has been proven [153, 164, 232, 323]. Together, these anatomical, connectional and functional data demonstrate that:

The SCN of humans and rats is similar

Importantly, although the SCN itself is similar, its effect on physiology and behavior is the reverse for night- and day active animals. The difference in SCN-output is discussed in “CLOCK OUTPUT”, page 22. The advantage of using the rat (nocturnal) - although it is not the best model for a human (diurnal) - is explained in “THE SCOPE OF THE THESIS”, page 33.

B. CLOCK INPUT

Blind people lack the synchronizing input from light to the SCN and consequently most of them show a free-running rhythm with a period longer than 24 hours. Periodically the endogenous circadian rhythm of these people shifts in and out of synchrony with
the external day-night rhythm. This results in a periodically distorted sleep-wake rhythm and a periodical inability to adapt to the social day-night rhythm. For example, with a free-running period of 25 hours, sleeping problems will occur, disappear and recur in a cycle of 24 days. In the absence of light (the day-signal and the most important input to the SCN), melatonin, the night-signal and probably the second strongest input to the SCN, can entrain these people if it is taken repeatedly at the preferred time of sleep [193, 280]. However, some completely blind people do not free-run. It seems that these people have an endogenous circadian rhythm close to 24-hours [192], in which case weaker entraining factors like food intake and activity may be sufficient to maintain entrainment to a 24-hour day-night cycle. Furthermore, there are also “visual cortex blind” people, who still show suppression of melatonin when they are exposed to light, which demonstrates that there are different pathways and different systems for light detection [69]. The multitude of influences on the circadian system may be the cause of disturbance when the timing is wrong (light during the day in night-shift workers), but may also provide new ways of treatment of circadian disturbances when the timing is right.

Light: circadian photoreceptors

There are two modes of photoreception. Firstly, there is visual photoreception, which provides us with a spatio-temporal image of the environment. Secondly, there is nonvisual photoreception, which affects the circadian system. In mammals, light affects the circadian system via the projection from the retina to the SCN. However, which photoreceptors are responsible for circadian photoreception? In the past decade rapid progress has been made in this quest. Recently, it was demonstrated in rodent and human that the 3-cone system and the rods, the visual photoreceptors, are not required for transmitting the light signal to the circadian system [37, 196, 330]. Furthermore, a distinct set of ganglion cells in the inner retinal layer were demonstrated to project to the SCN [233]. Retinal ganglion cells were thought only to pass on information from the rods and cones. However, it was recently demonstrated that the subset of these retinal ganglion cells that project to the SCN is itself sensitive to light. Only those ganglion cells that project from retina to SCN selectively contain the newly discovered vitamin-A based photoreceptor molecule melanopsin [112, 260]. Furthermore, studies determining the action spectrum show that blue-green light (450-500 nm) is the most potent in shifting the phase of the circadian rhythm [37, 230, 321, 330]. This action spectrum matches the sensitivity peak of melanopsin. Together, this indicates that, within the specific set of retinal ganglion cells, melanopsin is (one of) the crucial circadian photoreceptor molecules. Be that as it may, it could still be that additional photoreceptor molecules participate in circadian photoreception systems, resulting in more redundancy of the circadian photoreceptor system [298].

Although circadian light perception by the eye is generally acknowledged, there are
reports of extraocular light perception in mammals. Illumination of the back of the knee was reported to cause a clear circadian phase shift in humans [58]. Furthermore, it was reported that in anophthalmic mutant rats (without eyeballs), light could suppress melatonin levels [140]. However, no other research group could replicate the effects of extra-ocular light on the circadian system in mammals (unlike in non-mammalian species). Three independent research groups demonstrated that extraocular light was incapable of suppressing melatonin in man [127, 144, 194]. Also in Syrian hamster there was no suppression of melatonin, nor a phase shift as a result of extraocular light [216, 370]. Finally, after careful repetition of the conditions used by Campbell and Murphy, three independent research groups did not find an influence of extra-ocular light on the phase of the circadian rhythm in humans [82, 168, 187]. Most scientists of the chronobiological community agree that there is no support for extra-ocular light affecting the mammalian circadian system (see [98] for review). With the publication of Wright and Czeisler in Science, showing that light behind the knee (and carefully controlled darkness of the eye) was unable to either phase-shift or suppress melatonin [368], the less careful study by Campbell and Murphy in Science seems to be contradicted sufficiently.

**Light: immediate excitation of SCN**

Light is the most important input to the SCN, and acts as the signal of the day. Light has two effects on the SCN, causing 1) immediate excitation of SCN-neuronal activity and 2) a phase shift in the circadian rhythm of the SCN. In this part (Light: immediate excitation of SCN, page 17), the immediate effect of light is discussed, and in the next part (Light: long-term effects on circadian rhythm of SCN, page 19), phase shifting by light is discussed.

To receive light input, the SCN is conveniently located on top of the optic chiasm, from which it receives direct neuronal projections originating from the retina [149, 184]. This direct pathway from the retina to the SCN is called the retino-hypothalamic tract (RHT). The dispersed array of retinal ganglion cells containing melanopsin probably makes up an important part of the RHT, which signals light information to the SCN [125]. Light stimulation of the retina results in direct secretion of the excitatory neurotransmitter glutamate from the RHT into the ventral, VIP-containing, part of the SCN [80, 222, 352]. In a sub-population of the glutamate-containing retinal ganglion cells, pituitary adenylate cyclase-activating polypeptide (PACAP) is colocalized, which is also involved in relaying light information to the SCN and may potentiate the effect of glutamate on the SCN [120, 225].

Apart from the direct pathway via the RHT, which is the most important pathway to convey purely photic information, there is also an indirect pathway via which the SCN is informed about both photic and nonphotic stimuli. Light information via this indirect photic pathway is sent from the retina, via NPY-immunoreactive neurons in the IGL, to
the ventral part of the SCN [256]. The projection from the IGL to the SCN is called the geniculo-hypothalamic tract (GHT). Under LD conditions, but not under DD conditions, NPY levels in the SCN show a daily rhythm, with two peaks corresponding to the transitions of D-L and L-D. The overlap in SCN projection areas of the RHT and the GHT suggests that the GHT is involved in modulating the circadian response to photic input. Although the IGL has no clear influence during 12:12 LD conditions or constant light conditions, the IGL seems required for entrainment of the circadian system to a skeleton photoperiod, relevant for burrowing rodents [84]. The projections from the IGL are also involved in non-photonic signalling to the SCN (see “Other nonphotonic inputs to the SCN”, page 21).

Generally, little attention is paid to the immediate effect of light on SCN-neuronal activity and SCN-output. In vivo, nocturnal light exposure of the retina results in an immediate increase in the activity of the majority of SCN neurons [212,218]. In vitro, it was shown that glutamate, the main neurotransmitter released from the RHT, immediately stimulated SCN-neuronal activity in the majority of cells [211]. Both in nocturnal and diurnal animals, light causes stimulation of the SCN towards daytime levels (see intermezzo “Exciting Light”, below). Not only the activity of the SCN itself is stimulated by light, but also the output of the SCN is influenced immediately, which is discussed in “Immediate effect of light on SCN-output”, page 31. In this way, light signals ‘day’ to the rest of the body, as hypothesized in “Immediate effect of light on SCN-output”.

**EXCITING LIGHT**

Although nocturnal light exposure increases neuronal firing rate when measuring multi-unit activity of the SCN, not all individual SCN-neurons increase their firing rate during light exposure [214, 218]. Of the light-responsive SCN neurons in the rat, 65-85% increase their firing rate and 15-25% decrease their firing rate [218]. In diurnal animals investigated, the ratio of excited and inhibited SCN neurons is in favor of inhibition. In the thirteen-lined ground squirrel, using a modified recording method, 53% of the photically responsive SCN neurons were light-suppressed [214]. In a comparative study using the rat and the diurnal Octodon degus, 73% of responsive SCN neurons of the O. degus were light-suppressed [145]. At first glance, this seems to indicate that, while light in nocturnal animals is excitatory for the SCN, light is inhibitory in diurnal animals. However, this does not seem to be the case. The increases in firing rate measured after light exposure are caused by glutamate release from the RHT onto neurons mainly in the ventral part of the SCN. Because the inhibitory neurotransmitter GABA is ubiquitous within the SCN, stimulation of those SCN neurons by light is likely to lead to GABA release from those neurons onto other SCN neurons. Consequently, the firing rate of those ‘second order’ neurons will decrease. Thus the difference in the amount of SCN neurons being excited and inhibited depending on the species, indicates a quantitative difference in response to light rather than a qualitative difference. In other words, light is expected to induce a similar effect on the SCN in different species.
Light: long-term effects on circadian rhythm of SCN

Next to the immediate excitatory effect of light on the SCN, light can also phase-shift the SCN and thereby entrain physiology and behavior to the LD cycle. The effect of light depends on the circadian phase exposure: light at the beginning of the (subjective) night causes a phase delay, while light at the end of the night causes a phase advance. This is true for both night- and day-active animals [226, 277, 339]. A large part of the molecular mechanism that explains the phase-shifting effects of light has been revealed during the last few years. Exposure of an animal to light in the (subjective) night period first induces the expression of immediate early genes (IEGs) such as c-fos, B-fos, jun-B [13, 279]. IEG-expression is first induced in the ventral part of the SCN and subsequently in the dorsal part [272]. Therefore, it is believed that light information first stimulates the neurons in the ventral part of the SCN, containing mainly VIP, and that these neurons, in their turn, stimulate the neurons in the dorsal part of the SCN, containing mainly VP. The IEG expression is the first step to activate the molecular clockwork. The mechanism of the time-dependent phase-shifts of the circadian rhythm is illustrated in Figure 1. Per mRNA (mPer), an important player in the molecular clock, has an endogenous circadian rhythm in the SCN. mPer decreases at the beginning of the subjective night and increases at the end. Light at night increases Per1 and Per2 mRNA and protein expression [303]. An increase of mPer at the beginning of the night will thus delay the decrease in mPer and cause a phase delay. Increasing mPer at the end of the night will enhance the endogenous increase of mPer and thus advance the clock. During the day, when Per is already high, light has no effect on the phase of the molecular feedback loop (therefore,

Fig. 1 Explanation of the phase-shifts caused by light early or late at night. The molecular clock protein Per1 follows an endogenous circadian rhythm of expression in the SCN, with a minimum during the (subjective) night, and a maximum during the (subjective) day. Light increases Per1 expression. Light in the early night therefore delays the decrease in Per1 levels, and thereby delays the molecular clockwork of the SCN. On the contrary, light at the end of the night period accelerates the increase in Per1 levels, thereby phase-advancing the circadian rhythm. Light during the middle of the day has no effect, since endogenous Per1 levels are already high at that moment.
the day is called "the dead zone"). That the increase in Per is crucial for the phase shifts has been demonstrated by the repression of phase shifts by inhibition of light- or glutamate-induced Per expression [4]. If the rhythm in Per level were the only factor determining the size of a phase shift, phase shifts of 12 hours would be possible. However, because the other players in the molecular clock, e.g. Per3, CRY, and Clock, are not affected by light, the isolated change in the rhythm in Per causes a 'central jet lag'. The resultant phase shift is thus much smaller than could be expected on the basis of Per alone. Furthermore, the phase shift of the overt circadian rhythms, like locomotor activity, is not immediate. After a light pulse at night, there is a transient period of a few days during which overt rhythms slowly adapt to the new phase. This may be due to the 'central jet lag' within the SCN. Additionally, slow adjustment of the peripheral clocks to the new output of the master oscillator may slow down the phase adjustment even further (see Discussion “Synchronization of peripheral clocks”, page 149).

**Melatonin: immediate inhibition of SCN**

Melatonin is used worldwide against jet lag. The reason for this is two-fold and pertains to the double function of melatonin on the SCN. Firstly, melatonin can phase-shift the circadian rhythm of the SCN, which can accelerate the adaptation of the circadian rhythm to a new day-night rhythm. Secondly, melatonin has an immediate inhibitory impact on the SCN, which can accelerate falling asleep in humans at circadian phases that would otherwise be difficult [81]. In this part, I will discuss the immediate effect. I will discuss the long-term effect of melatonin on the SCN in the next part.

The most important target of melatonin is the SCN, with the highest density of melatonin receptors in the brain [269]. Furthermore, while the binding of melatonin in different brain structures varies greatly for the various mammalian species, melatonin is invariably bound in the SCN. Melatonin, as signal of the night, immediately suppresses the activity of SCN neurons towards nighttime levels, inhibiting neuronal firing by hyperpolarization [340], and lowering VP secretion [358]. By doing this, melatonin 'provides a level of security' against unwanted stimuli at night to activate and shift the SCN [22]. Light, on the other hand, has a double role, since it does not only lead to glutamate release onto the SCN, but also suppresses melatonin levels, thereby allowing light to phase-shift the SCN and to increase SCN-neuronal activity. The protective effect of melatonin is clearly demonstrated during continuous light exposure (LL) for many days, which distorts circadian rhythmicity. Under these conditions, daily melatonin application during the subjective nights can secure the SCN against constant stimulation and restore the disturbed circadian rhythmicity [78].

Nearly 100% of melatonin receptors in the brain are of the Mel1a subtype. Mel1a receptors are essential for “silencing” the SCN [188]. However, in mice without Mel1a receptors, the phase shifting effects of melatonin are only modestly altered [188]. In ham-
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A major breakthrough in understanding circadian rhythms is the discovery that melatonin, a hormone produced by the pineal gland, plays a crucial role in phase shifting and other aspects of circadian function. Mice genetically engineered to lack a functional Mel1b receptor, the phase shifting is completely un-attenuated [360]. All this suggests that in intact mammals the Mel1a is the main receptor for both melatonin-induced phase shifts and neuronal inhibition, but that in Mel1a knock-out mice, the low levels of Mel1b receptor in the SCN can take over the phase-shifting function of Mel1a [188]. The regulation of melatonin secretion as output of the SCN is discussed in "Melatonin regulation by SCN", page 28.

Melatonin: long-term effect on circadian system of SCN

The second effect of melatonin is shifting the phase and influencing the amplitude of the circadian rhythm of the SCN over several cycles. That melatonin indeed has the phase-shifting effects via the SCN, is demonstrated by phase-shifting of the SCN in vitro by melatonin [107]. If used correctly, melatonin can accelerate the adaptation of the circadian system to the new day-night rhythm of the destination. Melatonin intake in the evening can phase-advance the rhythm, while in the morning it can cause a modest phase delay. The endogenous plasma melatonin levels start to rise some 2 hours before sleep to reach maximum levels during sleeping hours. If this melatonin peak is advanced by intake of melatonin before the endogenous melatonin peak, this results in a phase-advance of the SCN and in this way of the circadian rhythms [171, 282]. Repeated melatonin application has proven efficient in synchronizing the endogenous circadian rhythm, not only in the case of jet lag, but also in people with disturbed circadian functioning, such as blind subjects [193, 280], patients with dementia [227], shift workers [281], and even healthy controls [12].

Not only can repeated nighttime melatonin synchronize circadian rhythms, it can also amplify and even restore circadian rhythms in human and rat [169, 376]. Daily melatonin can even restore a circadian rhythm that is completely absent due to continuous illumination [78]. In rats, daily melatonin application also amplifies the circadian rhythm of pineal norepinephrine content in rats [250], indicating an amplification of the sympathetic output of the SCN to the pineal gland. That repeated nighttime melatonin can amplify general SCN output is indicated in aged rats that have reduced circadian rhythm amplitude in sympathetic ganglia activity, heart and adrenal. Repeated nighttime melatonin partly restored circadian functioning in all aforementioned ganglia and organs [41]. This effect of melatonin to amplify circadian output of the SCN was used in Chapter 8.

Other nonphotic inputs to the SCN

Apart from melatonin, there are also other non-photic signals to the SCN, like locomotor activity and arousal. Like melatonin, locomotor activity can have both phase-shifting and immediate effects on the SCN. However, the effects of nonphotic stimuli are the opposite of the effects of light on the SCN, concerning both phase adjustments and im-
mediate effects. The phase response curve for locomotor activity is roughly the same as that of light, but 180° out of phase, with phase advance by novel wheel running during the day and no effect during the night [27, 234, 235]. Also, the immediate effect is the opposite to that of light, with behavioral activity inhibiting SCN neuronal activity and reducing c-fos expression in the SCN, while light increases both [215, 217, 223]. These effects appear to be partly mediated via the IGL, since lesioning prevents phase shifting to arousal (but not to light) [124]. This is supported by the fact that the application of NPY (the neurotransmitter secreted from the IGL in the SCN) on the SCN in vitro mimics the phase response curve of a novel wheel [121]. Another important pathway to relay nonphotic information to the SCN is serotonergic (5-HT) input from the median raphe nucleus [228]. Also here, the effects are opposite to those of light, with a reduction of Per1 and Per2 in the SCN by 5-HT (in contrast to the increase by light) [135]. Nonphotic stimuli can also counteract the effect of light on the SCN, with NPY and 5-HT inhibiting light-induced phase shifts, light-induced SCN-neuronal activity, and light-induced c-fos expression [257, 265, 371, 372]. That nonphotic signals are also important in human entrainment has been demonstrated in completely blind subjects who were without light input into the SCN [166].

C. CLOCK OUTPUT

A clock without hands is of no use. The molecular clock signal of the SCN needs to be passed on to the rest of the body. Parts of the molecular clock should affect the expression and secretion of neurotransmitters and the membrane-electrophysiological properties of the SCN neurons, to cause a rhythm in SCN-output. Although these mechanisms are largely unknown, an example of an effect of clock genes on output genes is provided by the regulation of VP. The CLOCK-BMAL1 complex, which is expressed in a daily rhythm, directly activates VP transcription, and could thus lead to the circadian production of VP in the SCN [146]. However, not transmitter production, but transmitter release determines the output signal of the SCN. Since transmitter release is determined by the electrophysiological properties of the neuron, the critical question is how the molecular clockwork regulates the electrophysiological condition of the SCN neuron. Although it is suggested that the clock genes may affect the expression or post-translation of ion channels to generate day-night rhythms in electrical activity of SCN neurons [252], the exact mechanisms are as yet unknown, but will hopefully be revealed in the near future.

SCN-output
Transplantation of the SCN to an arrhythmic donor can restore the circadian rhythm in
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locomotor activity. Inspection of the grafted tissue revealed that hardly any neuronal outgrowth to the rest of the brain was present, suggesting that a humoral factor may pass on the signal of the SCN. This was investigated by wrapping the SCN in a semi-permeable coating and placing it back into the brain [307]. This prevented neuronal outgrowth and only diffusible substances could communicate the signal of the SCN. In these SCN-encapsulating studies again the rhythm in locomotor activity was restored [180, 220]. However, hormonal rhythms, like those of melatonin and cortisol, could not be restored. This demonstrates that the neuronal projections of the SCN are crucial to the communication of all signals from the SCN.

The neuronal projections of the SCN to other brain areas within and outside of the hypothalamus are extensive. Via these brain areas, the SCN influences all important organs of the body via neuronal (autonomic nervous system) and endocrine mechanisms. Within the hypothalamus, the SCN has massive projections to the dorsomedial hypothalamus (DMH), paraventricular nucleus of the hypothalamus (PVN), sub-PVN, lateral hypothalamus (LH), and medial preoptic area of the hypothalamus (MPOA). Outside of the hypothalamus, the SCN projects to the paraventricular nucleus of the thalamus (PVT). The neurotransmitters of these projections include VP, VIP, GABA, and glutamate. Vasopressinergic SCN neurons regulate different circadian hormone rhythms. VP release in the DMH, probably via GABAergic projections to the PVN, suppresses CRH release in the anterior pituitary, and this consequently leads to suppression of corticosterone concentration in the plasma [47, 152]. Vasopressin released from the SCN in the MPOA on the other hand, stimulates GnRH release and thereby the release of luteinizing hormone [247]. GABA and glutamate are also important for SCN output, and are, for example, involved in the regulation of melatonin, and possibly heart rate (HR), as will be discussed further in subsequent parts of this thesis.

The SCN has a circadian rhythm in general electrical activity, with high levels during the (subjective) day and low levels during the (subjective) night. But how could a single circadian rhythm in SCN activity result in the complex diversity of the many circadian rhythms of different organ systems? The many rhythms differ in their shape and with respect to the time at which they peak (Fig. 2). In humans, melatonin shows a wide peak (or plateau) at night, cortisol has a peak in the early morning and BT follows a more sinusoidal rhythm with a peak in the afternoon. Although additive down-stream processes are likely to be involved, there are two important aspects of SCN functioning that could enable the required differential output that results in the various circadian rhythms of our body:

1. TRANSMITTER DIFFERENTIATION

If all SCN-neurons had the same activity rhythm with a peak in the middle of the day, different neurons could use different neurotransmitters (or receptors in the target) to either activate or inhibit a target area. Such a mechanism could help explain how general
Fig. 2 Schematic representation of three circadian rhythms in humans. 1) The rhythm in melatonin (small dotted line) has a wide peak during the night and very low levels during the day. 2) The rhythm in cortisol (continuous line) shows the lowest levels in beginning of the night, an increase starting halfway the night, and a peak after wakening. 3) The rhythm in body temperature shows peak levels at the end of the day, decreases rapidly at sleep onset and reaches its minimum some 3 hours before waking, after which the ascent starts. The diversity in shapes of circadian rhythms requires differential output of the SCN (see "CLOCK OUTPUT", page 22).

stimulation of the SCN results in both an exciting (glutamate) and inhibiting (GABA) signal to single PVN neurons [67]. Furthermore, within single SCN neurons different neurotransmitters are colocalized. Neuropeptides (e.g. VP and VIP) require a higher rate of depolarizations for their release than other neurotransmitters (e.g. GABA and glutamate). Thus the strength of excitation of a neuron could result in the release of a single neurotransmitter or of more neurotransmitters from the SCN to its target areas, thereby enabling a more complex output from a single neuron. However, if all SCN-neurons had the same peak in transmitter secretion during the middle of the day, this could only explain the simultaneous stimulation of one organ and the inhibition of the other. However, it could not explain the early morning peak in cortisol in humans (or the early night one in rats). This indicates the presence of another differentiation within the SCN.

2. REGIONAL DIFFERENTIATION
SCN-neuron populations with different peaks in activity would allow for more complex regulation of circadian outputs, e.g. the cortisol rhythm. Recently several regional differences in the SCN have been demonstrated, comparing the left and right half [137, 289], the anterior with the posterior part, and the ventrolateral with the dorsomedial part [295], but also on a smaller scale (Chapter 8, Thesis of Schaap J). The study of SCN neuronal activity with higher resolution, and linking SCN-regional differentiation with different circadian rhythms (as melatonin, cortisol and temperature) will reveal if there are specialized parts of the SCN for different circadian outputs.
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Different SCN-output in diurnal and nocturnal mammals

In both diurnal and nocturnal mammals, general SCN electrophysiological, metabolic and transcriptional activity is highest during the day. It would thus be expected that high general SCN activity causes daytime physiology and behavior in all mammals. Therefore, the effect of SCN activity on physiology of diurnal and nocturnal mammals would be expected to be each others opposite.

How could the SCN have an opposite effect in diurnal and nocturnal species? Since there are both stimulatory and inhibitory neurotransmitters, active SCN-neurons could either stimulate or inhibit their target. If a SCN projection to a brain structure used GABA (inhibitory) in rats and glutamate (excitatory) in humans, a similar activity rhythm of the SCN-neurons would result in the opposite physiological or behavioral rhythm.

One important exception to an opposite circadian rhythm is melatonin. Melatonin is always high during the (dark) night and low during the day, independent of the mammalian species. The only other known example is leptin; a hormone secreted by white adipose tissue, which signals the metabolic state and is also high at night in rat and human. This could imply that these hormones have an opposite function in day- and night-active animals. On the other hand, the rhythms in melatonin and leptin are similarly linked to the activity of the SCN: high levels when general SCN-activity is low (night), and *vice versa* (day). Therefore, these hormones may have a similar effect on the SCN, as has been discussed for melatonin (see "Melatonin: immediate inhibition of SCN", page 20 and "Melatonin: long-term effect on circadian system of SCN", page 21).

Circadian regulation by autonomic nervous system

Generally, the sympathetic nervous system is responsible for the emergency reaction, or *fight or flight* response, preparing the animal for behavioral activity [59]. The parasympathetic nervous system is generally responsible for *rest and digest*, decreasing energy use and increasing energy intake. However, can general sympathetic activation explain the opposite local effects, such as stimulation of the heart but inhibition of the gastrointestinal system, and vasoconstriction in the face but vasodilatation of vasculature of the lungs? The autonomic nervous system uses three mechanisms to differentiate its effects on the body: differentiation in receptors on the target tissue, differentiation in neurotransmitters secreted by autonomic nerve endings, and differentiation in local activity of autonomic projections.

1. RECEPTOR DIFFERENTIATION

The same neurotransmitter can have the opposite effect, depending on whether the receptor has an excitatory or inhibitory effect on the cell. Norepinephrine from the sympathetic nervous system can cause increased HR, vasoconstriction in most arteries and increased sodium uptake by the kidney, but at the same time it can cause vasodilatation in bronchial, coronary, and muscular arteries, and decrease the activity of the
gastrointestinal tract. Likewise, acetylcholine from the parasympathetic nervous system inhibits the heart, but at the same time activates the gastrointestinal system. These differential effects of a single neurotransmitter require differential postsynaptic responses. Indeed, multiple receptors are present for norepinephrine and epinephrine with different effects. α1-Adrenergic receptor activation causes vasoconstriction of resistance vessels but α2-adrenergic stimulation causes vasorelaxation via negative feedback. β1-Adrenergic receptor stimulation has a chronotropic (increase in HR) and ionotropic (increase in stroke volume) effect on the heart, while β2-adrenergic receptor activation leads to vasodilation in heart and muscle (allowing more metabolic activity), and β3-adrenergic excitation activates uncoupling protein, leading to increased heat production. At least three muscarinic receptors are known, of which M1 causes stimulation of the gastrointestinal system and M2 causes inhibition of, e.g., the heart. In this way a single neurotransmitter can cause vasoconstriction in one part of the peripheral vasculature and vasodilation in the other.

2. TRANSMITTER DIFFERENTIATION

Also by using a different neurotransmitter, a neuron may have the opposite effect on a target. Furthermore, multiple neurotransmitters are colocalized within single autonomic neurons. Transmitters besides norepinephrine, present within sympathetic neurons are suggested to include NPY and ATP, of which NPY causes vasoconstriction [199]. On the other hand, sympathetic projections can also contain neuronal NOS, which may explain vasodilation of vasculature by sympathetic stimulation [74]. Thus, through transmitter differentiation, sympathetic stimulation can cause either vasoconstriction or vasodilation.

If only these two mechanisms were used, there could only be gradients in overall activation of the body. However, except for the extremes with general sympathetic or parasympathetic activation, more local regulation should take place because of differing local demands. For example, a change in autonomic balance to the pupil in favor of the parasympathetic nervous system, causing pupillary constriction in response to light, should not automatically lead to an increased gastrointestinal motility, decreased HR, and blushing of the face. Therefore, regional differentiation of the autonomic nervous system would be required to allow for such a local control. Also for the circadian system, local regulation should take place because of varying demands of different organ systems at different times of the day, to explain circadian rhythms as diverse as the nocturnal melatonin, morning cortisol and afternoon temperature peak in humans.

3. REGIONAL DIFFERENTIATION

On large scale, this required regional differentiation is known for the sympathetic and parasympathetic nervous system, which are divided into different segments, as exemplified by the different sympathetic ganglia. Further proof of the importance of regional differentiation in autonomic output per organ comes from tracing experiments. Regional
differentiation within the spinal cord in the regulation of heart and adrenal is suggested by retrograde tracing of the stellate ganglion and the adrenal cortex, which leads to only 1% of neurons in the IML showing colocalization [261]. Even different fat pads (subcutaneous versus intra-abdominal) are regulated by different neurons in the IML [173].

On the other hand, Jansen and coworkers showed that in higher brain regions (e.g. PVN), single neurons do project to both the adrenal cortex and the stellate ganglion [141]. However, it was demonstrated recently by the autonomic differentiation between sympathetic and parasympathetic remaining present up to the level of the SCN that regional differentiation is even present in higher brain structures like the SCN (Buijs RM, personal communications).

Therefore, I would like to hypothesize that the combination of the regional outflow of the SCN (as discussed before) to different target areas, and the regional output via these target areas to the sympathetic nervous system, together would allow the complex task of transmitting completely different autonomic signals for optimum preparation of the organism for circadian changes.

Let us discuss the role of the aforementioned three mechanisms in the differential circadian regulation of melatonin, BT and cortisol. Melatonin secretion by the pineal is only regulated by the sympathetic nervous system and this effect is stimulating, thus sympathetic output to the pineal should be *high at night*. The endogenous circadian rhythm in BT is regulated by both the endogenous circadian rhythm in heat production and heat loss [172]. Heat production is for an important part caused by activation of uncoupling proteins. Sympathetic activity increases heat production by uncoupling proteins. Although thyroid hormones also play an important role in stimulation of uncoupling proteins, they cannot explain the rhythm in heat production because thyroid hormones have their peak at night, when temperature is low in humans. Thus, with the endogenous circadian rhythm in heat production having a peak during the day, sympathetic stimulation to uncoupling proteins (UCP) is expected to be *high during the day*. Heat loss is for an important part regulated by vasoconstriction/vasodilatation of the distal skin. Sympathetic activity causes vasoconstriction, and thus reduces heat loss. Since heat loss is minimal during the subjective day, sympathetic activity to the vasculature of the distal skin is also expected to be *high during the day*. For the adrenal cortex it has been indicated that sympathetic stimulation in the morning increases the sensitivity of the adrenal cortex to ACTH, resulting in the peak of cortisol concentration in the morning (see "Corticosteroid regulation by SCN", page 28). Sympathetic output to the adrenal cortex is thus expected to be *high in the early morning*. The peak in sympathetic output to the pineal at night, to UCP and peripheral vasculature of the skin during the day, and to the adrenal cortex in the morning therefore illustrates that these different circadian rhythms are caused by *regional differentiation* in sympathetic output and are not the result of a difference in receptor subtype or a difference in co-transmitter secretion to cause sympathetically mediated inhibition.
Corticosteroid regulation by SCN

Corticosteroids show a clear endogenous circadian rhythm in mammals. Traditionally, it was thought that the circadian rhythm in corticosteroids is caused by the rhythm in ACTH levels. However, injection of the same doses of ACTH in dexamethasone-treated or hypophysectomized rats (both without endogenous ACTH rhythm) increases corticosteroid levels 2.5 times more at the beginning of the active period than at the beginning of the resting period, suggesting that extra-ACTH mechanisms play a significant role in the (circadian) regulation of the adrenal cortex [72, 245]. The magnitude of the daily rhythm in ACTH-sensitivity was greater than that in ACTH levels, indicating the importance of the extra-ACTH mechanism [72]. What could this extra-ACTH mechanism be?

In hypophysectomized dogs whose physiological concentrations of ACTH were replaced, it was demonstrated that stimulation of the (sympathetic) splanchnic nerve causes an increase in cortisol levels [90]. Furthermore, it was demonstrated that the effect of sympathetic stimulation was not caused by the increase in adrenal blood flow, and thus in ACTH presentation rate, because the cortisol increase preceded the change in ACTH presentation rate [90]. The autonomic nervous system also plays a crucial role in the control by the SCN of the adrenal cortex. Projections from the SCN to the PVN area not only modulate corticosterone levels by means of the traditional, hormonal hypothalamus-pituitary-adrenal axis (HPA-axis) as mentioned before, but also via the autonomic nervous system [47]. The multisynaptic autonomic projection for this effect has been demonstrated with the use of pseudorabies virus (PRV) injections. After injection, PRV is transported retrogradely and selectively across synapses [60]. Injection in the adrenal cortex results in PRV first-order staining in the IML, second-order staining in the autonomic PVN (e.g. oxytocin neurons) and third-order staining in the SCN (VP and VIP neurons) [47]. Apart from the inhibitory influence of VP on corticosteroid secretion, there is also an unrevealed excitatory signal that is derived from the SCN [159].

Melatonin regulation by SCN

GABA release from SCN projections in the PVN area is critically involved in both the circadian rhythm in melatonin and the light-induced suppression of melatonin. The GABA release in the PVN area inhibits the sympathetic IML, thereby suppressing, via the superior cervical ganglion, norepinephrine release in the pineal gland. This multisynaptic autonomic pathway has been demonstrated using the PRV technique [324]. Sympathetic stimulation of the pinealocytes increases both melatonin production and release. Production is increased by activation of the rate-limiting enzyme NAT. The circadian rhythm in melatonin is the result of GABA release from the SCN into the PVN during the subjective day, without GABA release during the night. This leads to suppression of melatonin secretion during the day, while the absence of GABA during the night
allows high melatonin levels at night. At night, light stimulation of the SCN causes GABA-release from the SCN into the PVN and results in the immediate suppression of melatonin production and secretion [153, 154]. Nevertheless, we have preliminary evidence that other transmitters from the SCN are also involved in the regulation of melatonin secretion. Glutamate release from the SCN into the PVN seems to be crucial for melatonin to reach nighttime peak levels (Perreau S, personal communications). It thus seems that the SCN regulates melatonin secretion by both an inhibitory signal and an excitatory signal, just like in the control of corticosteroid secretion.

**Temperature regulation by the SCN**

During everyday life, our BT has a daily rhythm with a day-night difference of about 1°C. About 0.4°C is of an endogenous nature, while the rest is caused by the daily rhythm in behavioral activity [172]. BT is often recorded as an indicator of the phase position of the circadian rhythm. Even under ambulatory conditions, the nocturnal temperature minimum seems a reliable estimate of the phase of the endogenous circadian rhythm [171]. An example of the temperature rhythm in a healthy young man at night is shown in figure 3. The involvement of the biological clock in this temperature rhythm is further illustrated by the ability of light to shift the phase of the rhythm [68].

However, there is great controversy on the question whether the SCN is the only circadian pacemaker in temperature regulation. Some studies report the complete absence of a daily rhythm in BT after SCNx [3, 83, 267, 276], while other results suggest that the SCN is not required for the circadian rhythm in BT [286, 353]. To investigate if the endogenous BT rhythm is produced by the SCN or by another structure, animal experiments are required. However, investigating the endogenous rhythm without the effect of the day-night rhythm in behavioral activity is difficult in animals. Obviously, it is impossible to measure the circadian rhythm in BT during 24 hours without activity in conscious and unstressed (unrestrained) rats. Previously, indirect methods have been used in an attempt to calculate the endogenous circadian rhythm in BT from ambulatory data. However, such mathematical methods are unable to estimate this rhythm reliably because of the erroneous assumptions of fixed temperature effects of certain activity levels and of a sinusoidal shape of the BT rhythm. Alternatively, in the present thesis, we used a direct method by selecting voluntary resting periods of sufficient duration to prevent the effect of previous activity on BT (Chapter 3). The role of the SCN and the control of both heat production and heat loss will be discussed in “Thermoregulation by the SCN, involvement of vascular system” of the Discussion.

**Sleep and activity regulation by the SCN**

One of the most obvious but least understood circadian rhythms is the organization of the sleep-wake rhythm. We spend nearly one third of our lifetime asleep - not a waste of
Fig. 3 Example of the rhythm in BT, with a minimum at ZT21, 3 hours before the habituated waking time of the subject (ZT0; 6:30), typical for the endogenous circadian temperature rhythm (upper figure). The night after a 1-h 1300-lux light exposure in the evening (ZT15-16), the temperature minimum is delayed by 2 hours to ZT23 (lower figure). Deep rectal temperature was recorded with 1-min intervals by Actiwatch-T (Cambridge Neurotechnology Ltd, Cambridge, UK) in a healthy young male (19 years), in a supine resting condition in a quiet, private hospital room. The temperature minimum was determined with a 1-h running average. Dark bar, sleeping period; open horizontal bar, supine but awake; vertical lines, 5-min visit to bathroom at ZT14, ZT16 and ZT24.
time, but crucial for life. Sleep deprivation results in a serious loss of cognitive function, decreased immune response [139], and ultimately death [92]. These and other findings have indicated that sleep may be physically and psychologically restorative.

Without SCN there are no circadian rhythms in wakefulness, rapid eye movement (REM) sleep, REM-associated behavior or non-REM sleep [85, 229, 317, 334]. The SCN also improves the quality of sleep and wakefulness, as illustrated by the reduced locomotor activity during the wake periods and the considerable reduction of deep sleep during the sleep periods in SCNX rats [334]. One brain area that has been strongly implicated in arousal and sleep-wake functions is the noradrenergic locus coeruleus (LC). Electrical activity of the LC is high during waking and low during sleep. Furthermore, stimulation of the LC leads to increased arousal as measured by EEG. The association between the SCN and the LC has been suggested only recently [15]. With the use of PRV, it has been demonstrated that the SCN has a multi-synaptic projection to the LC. The DMH is the most important relay station for this projection, as demonstrated by the elimination of the circadian rhythm in neuronal activity in the LC after lesioning the DMH. Next to the DHM, also the PVN, the MPO and the ventrolateral preoptic area (vPvPO) connect the SCN with the LC.

Immediate effect of light on SCN-output

Generally, the immediate effect of light on physiology and behavior is merely regarded as 'masking' of SCN-generated rhythms, a term first used by Aschoff [14]. Thus masking is mostly dismissed as merely obscuring the circadian rhythms under investigation. However, with the immediate impact of light on the SCN (see "Light: immediate excitation of SCN", page 17), it could be expected that light also affects SCN-output immediately. In the present thesis, the involvement of the SCN in known and novel effects of light on physiology is investigated. The best-known and most studied example is the suppression of melatonin secretion by nocturnal light, as illustrated in figure 4. The crucial role of the SCN in this effect of light on melatonin secretion has been demonstrated in rodents [164], while the same mechanism seems true for man [378]. Light information stimulates the release of GABA from the SCN in the autonomic parts of the PVN [153, 157]. Consequently, the sympathetic output from the PVN, via the IML and the superior cervical ganglion, to the pineal is inhibited [324]. Via a similar mechanism, light could be expected to also influence other organs via the autonomic nervous system. Indeed, there are several indications for a direct effect of light, via the SCN, on the autonomic nervous system [198, 238, 283, 304]. This is further dealt with in Chapter 2-6 and in "Pathways and transmitters used by SCN in regulation of the heart" on page 141 of the Discussion of this thesis.
Fig. 4 Example of the suppression of melatonin secretion by light in the evening in humans (left figure). Light does not influence melatonin in the morning, because at that time, melatonin secretion is already shut down, and the decrease is due to the half-life of melatonin of about 45 minutes (right figure). Data are obtained from the experiment conducted in the hospital setting as presented in Chapter 7. Please refer to that publication for more details.
With light exposure causing immediate stimulation of SCN-neuronal activity towards daytime levels both nocturnal and diurnal animals (see “Light: immediate excitation of SCN”, page 17), and the immediate effect of light on SCN-output, a central hypothesis for light in the present thesis is that:

*Light induces day-time physiology and behavior via the SCN, both in nocturnal and diurnal species*

**D. THE SCOPE OF THE THESIS**

The aim of the present thesis was to investigate if and how the SCN influences the cardiovascular system. The influence of the biological clock was investigated in two ways. Firstly, the endogenous influence of the SCN on the cardiovascular system was investigated. This was possible by recording under conditions that exclude the effect of “masking” factors such as behavioral activity and light. Secondly, the effect of stimulation of the biological clock on the cardiovascular system was investigated. Two stimuli of the SCN were used. Light was used as the day-signal and melatonin was used as the night-signal for the biological clock.

The present thesis is divided into studies conducted in rats (Chapters 2 and 3) and those conducted in humans (Chapters 4 - 8). There are three advantages in studying the circadian system in both rats and humans:

1. **OPPOSITE FUNCTION OF SCN IN NOCTURNAL AND DIURNAL ANIMALS**

The day-night rhythm in SCN activity is the same in night-active (nocturnal) and day-active (diurnal) mammals, with a peak during the day and a trough at night. The effect of SCN activity on the body in nocturnal animals is thus the opposite from that of diurnal ones. In the present thesis, this opposite influence of SCN activity on physiology in night-active (i.e. rat) and day-active (i.e. human) species is used as a tool to differentiate between startling or stressing stimuli and stimuli acting via the SCN on the rest of the body. A non-specific startle or stress stimulus will cause responses in the same direction in all species, while stimulation of the SCN will cause opposite effects in diurnal and nocturnal mammals. As mentioned in “Light: immediate excitation of SCN”, page 17, we hypothesize that

*Light induces day-time physiology and behavior via the SCN, both in nocturnal and diurnal species*

2. **EXPERIMENTAL FREEDOM IN RATS, COOPERATION BY HUMANS**

The second reason to do research on humans and rats is the experimental freedom in rats and the co-operative abilities in humans. For obvious reasons, physiological mechanisms can be investigated more invasively, and therefore more thoroughly, in rats.
humans, the role of the SCN in a process can only be indicated indirectly, while by eliminating the SCN in rats it can be proven directly. On the other hand, one cannot, for example, instruct a rat to remain at rest to study the HR independent of the disturbing influence of locomotor activity. In this respect, humans can voluntarily adapt to experimental confinements.

3. CLINICAL RELEVANCE
The third reason to include humans in the study is the obvious reason that a human is the best model for a human. Most animal experimental research, even fundamental research, is performed to learn more about humans, and finally to be applied to increase the health and prosperity of humanity. Research on humans has more direct clinical relevance.

The main question addressed in Chapter 2, is: does the SCN influence the heart? HR shows a clear day-night rhythm, allowing high metabolic activity during the active period and saving energy during the resting period. However, since HR is strongly influenced by behavioral activity, the question arises whether the daily rhythm in HR is directly regulated by the SCN, or a byproduct of the daily rhythm in locomotor activity. Since anticipation to coming activity and inactivity would allow the animal to be better prepared for the circadian demands, we hypothesized that the SCN influences HR independent of locomotor activity. To test this hypothesis, we used a new technique to analyze resting HR, independent of locomotor activity, during periods of voluntary rest in conscious, unstressed rats. We verified if a daily rhythm in resting HR would be a proper endogenous circadian rhythm in constant dark conditions (DD). Finally, we investigated if the SCN was crucial for a circadian rhythm in resting HR by bilateral lesioning of the SCN.

With our overall hypothesis "Light induces day-time physiology and behavior via the SCN, both in nocturnal and diurnal species", we furthermore hypothesized that nocturnal light exposure lowers HR towards daytime levels in rat, requiring the presence of a functional SCN. To test this hypothesis, SCN-lesioned and intact rats were exposed to light at night.

Thirdly, because the autonomic nervous system is most important in the regulation of HR, we hypothesized that the SCN has multisynaptic projections to the heart. Such a projection from the SCN to the heart could be the anatomical substrate for circadian and light-induced influences on HR. To test our hypothesis, we injected Pseudorabies virus (PRV), a retrograde transneuronal virus tracer, into the heart. Four days after injection, we investigated the hypothalamus for the presence of PRV within SCN neurons.

The main question of Chapter 3 is: does the SCN generate the circadian rhythm in BT? Controversy concerning the question whether there is a circadian oscillator outside of
the SCN in the regulation of BT in experimental animals remains in the literature. Behavioral activity has great influence on BT. Because of the adaptive advantage to anticipate changes in thermal demand, we hypothesized that the SCN influences BT, independent of locomotor activity.

To test this hypothesis, we used the same technique as in Chapter 2 to analyze BT without an influence of previous activity. The presence of an endogenous circadian rhythm was verified in DD. Finally, we investigated if the SCN was required for a circadian rhythm in resting BT, by testing the presence of a rhythm in SCN-lesioned rats. To investigate if the recovery time after SCN-lesioning could explain the controversy in the literature, we followed rats up to 6 months after lesioning.

Secondly, with our overall hypothesis “Light induces day-time physiology and behavior via the SCN, both in nocturnal and diurnal species”, we hypothesized that light exposure via the SCN lowers BT towards daytime levels in rat, depending on the phase of the day and on the intensity of the light, but independent of activity. To test this hypothesis, we exposed SCN-lesioned and intact rats to light at different phases of the day-night cycle, with different light intensities and by analyzing resting BT. To investigate if the light conditions during the experiments could explain the controversy in the literature, we compared SCN-lesioned rats during various light conditions (e.g. LL, DD, LL, etc.) up to 6 months after lesioning.

Previously, it was demonstrated that fasting increases the circadian rhythm amplitude of BT by lowering daytime temperature, without a change in nighttime temperature. Recently, it was demonstrated that in SCN-lesioned rats fasting has no influence on BT [191]. The SCN thus seems crucial for the effect of fasting on temperature regulation. Since we hypothesized before that the effect of light on temperature is mediated via the SCN, we hypothesized that light will have a stronger impact on temperature in fed rats, and will depend on the phase of the day. To test this, we exposed the same rats during fed conditions and fasted conditions to light at different times of the day, and compared the strength of the effect.

In Chapter 4, the main question is: is HR influenced by the SCN in humans? If the SCN regulates HR in humans, an endogenous circadian rhythm in HR would be expected. Therefore, it is hypothesized that human HR has a daily rhythm independent of external masking factors. To test this hypothesis, we repeatedly measured HR in healthy young males during awake, supine rest in the dark, after 2 hours of fasting, during normal working days. To further verify the reliability of this method to measure the endogenous circadian rhythm in HR, we compared these results with results from experiments performed under constant routine conditions, the golden standard to probe endogenous circadian rhythmicity in humans.

With our overall hypothesis “Light induces day-time physiology and behavior via the
SCN, both in nocturnal and diurnal species”, we further hypothesized that light causes a phase-dependent stimulatory effect on resting HR in human. Both a daily rhythm and a phase-dependent light-induced increase (as opposed to the decrease in rats) in resting HR would provide evidence for a role of the circadian pacemaker in the regulation of HR also in humans.

The main question in Chapter 5 is: what is the autonomic mechanism to explain the diurnal rhythm in HR and the light response of resting HR in human? We hypothesized that changes in parasympathetic cardiac outflow cause the diurnal rhythm and light response of resting HR. To test this hypothesis, we measured changes in pre-ejection period (PEP) as index of sympathetic cardiac activity, and changes in the root mean square of successive differences in inter-beat interval (RMSSD) as index of parasympathetic cardiac tone, over the day-night cycle and in response to light.

In animal experimental studies, it was previously demonstrated that light suppresses corticosterone levels depending on the phase of the day, and depending on the presence of a functional SCN [47]. Furthermore, it was demonstrated that this suppression in corticosterone was rapid and without a change in ACTH, suggesting of neuronal regulation [47]. This notion was supported by the delineation of a multi-synaptic projection from the SCN, via the sympathetic nervous system to the adrenal cortex [47]. The first question that arose was: what if the same were true for humans? Thus, in Chapter 6, the main question was: does the SCN influence cortisol secretion by the adrenal cortex in humans? With our overall hypothesis “Light induces day-time physiology and behavior via the SCN, both in nocturnal and diurnal species”, we further hypothesized that light causes a phase-dependent increase of cortisol in humans. This would be indicative of a role of the SCN. To test this, we measured the changes in cortisol levels with and without light exposure at two different circadian phases.

With the aforementioned proof of a role of the sympathetic nervous system in the effect of light on corticosterone in rats, the main question in Chapter 7 was, what is the role of the autonomic nervous system in the cortisol increase by light? We hypothesized that light increases cortisol levels independent of a change in ACTH levels. To test this, we measured salivary free cortisol, plasma total cortisol and plasma ACTH with a high sampling frequency in a hospital setting with and without light exposure during two different circadian phases.

Furthermore, we hypothesized that a hospital setting will slightly increase basal cortisol levels, but will not interfere with the stimulatory effect of light on cortisol. To measure the interference of experimental setup on cortisol levels, we compared the salivary cortisol levels of healthy young men at home and in a hospital setting.
Post-mortem studies of patients with essential hypertension demonstrated decreased neurotransmitter content of the SCN, a sign of disturbed SCN activity [110]. The main question of Chapter 8 was: is the SCN functionally disturbed in patients with essential hypertension, and what is the effect of supporting the circadian rhythm? Since the SCN influences the cardiovascular system (Chapters 2-5) and melatonin binds specifically to the SCN and can restore disturbed circadian rhythmicity, we hypothesized that *nighttime melatonin intake will lower blood pressure and amplify the circadian rhythm in patients with essential hypertension*. To test this, we studied the effect of 2.5 mg oral melatonin 1 hour before sleep on 24-hour blood pressure in a balanced, double blind, placebo-controlled, crossover-experiment. We studied both the immediate (single tablet) and middle-term (3 weeks) effect of melatonin. Simultaneously, we performed actography recording to estimate sleep quality. Ion is already shut down, and the decrease is due to the half-life of melatonin of about 45 minutes (right figure). Data are obtained from the experiment conducted in the hospital setting as presented in Chapter 7. Please refer to that publication for more details.