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Sleep deprivation and its impact on circadian rhythms and glucose metabolism

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SLEEP DEPPRIVATION AND ITS IMPACT ON CIRCADIAN RHYTHMS AND GLUCOSE METABOLISM

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SCOPE OF THESIS

1. Homeostasis and rhythms

Charles Darwin (1809-1882) demonstrated the considerable influence of environmental variations on life. Living beings on the Earth are subjected to various external changes (e.g. food availability, light, temperature, predation). Organisms therefore have to adapt to their environment in order to survive and maintain their own species. Contemporary to this, a new concept of internal processing and function was proposed by Claude Bernard (1813-1878). Bernard proposed the notion of the *interior milieu,* referring to the extra-cellular fluid environment, more particularly the interstitial fluid surrounding the organs of the individual. He suggested that maintaining the stability of the internal fluid (blood and other body fluids) is essential for the life of higher organisms, because multiple and/or chronic disturbances of this *interior milieu* would lead to pathophysiology. This concept has been redefined over decades and termed *homeostasis* (*Homeo* = same; *stasis* = steadiness) by Walter B. Cannon (1871-1945). Cannon defined homeostasis as "all organic processes that act to maintain the steady state of the organization, in its morphology and its internal conditions, despite external disturbances". In a more simple way, homeostasis is the ability of the body to maintain a state of relative stability of the different components of its internal environment despite the constant changes in the external environment. More recently, according to the Thermal Commission of the International Union of Physiological Sciences (IUPS; 2001), homeostasis is characterized by "the relative constancy of physiochemical properties of the internal environment of an organism as being maintained by regulation". In the body, homeostasis applies to many physiological processes such as the regulation of osmolarity, blood sodium level, plasma glucose, blood pressure, body temperature, feeding and sleep. It is of prime importance to note that physiological variables are not maintained constant in the absence of environmental perturbations and instead, display more or less marked rhythms, i.e., a relative constancy is maintained. Daily and seasonal changes in the environment are important variables that influence the homeostatic setting of the organism. However, the predictability of these changes also allows an organism to anticipate these environmental changes. The structures and mechanisms which are involved in the anticipation of these daily changes on their turn also interfere with the (constancy of the) internal environment. The interactions of both these processes, i.e., process 1 and process 2, and their consequences for the organisms' adaptive capacities encompass the subject of this thesis.

The 24-h period of Earth's rotation is correlated with major oscillations in many critical variables in the environment such as ambient illumination and temperature, the availability of

nutrients and activity of predators. The periodicity of these challenges and opportunities permits anticipation to these daily changes that in turn will shape the temporal organization of behavior and physiology of the organism. The internal temporal capability enables the organism to anticipate the probability of predictable demands upon the homeostatic system and therefore will decrease the homeostatic perturbation within a certain time frame and eventually reduce risks of disease. This feature is clearly illustrated with the rises in body temperature and plasma corticosteroid in advance of the time when animals awake from their daily sleep period. Waking itself may occur in advance of the time of lights-on in diurnal animals. Another example of physiological anticipation to daily changes is the increased level of plasma glucose in anticipation to glucose demands for the upcoming activity period, also called "dawn phenomenon" in humans (Bolli et al., 1984; Arslanian et al., 1990).

2. Rhythms of Life

The Earth's rotation around its axis generates daily environmental cycles. The daily environmental cycle of greatest importance for daily timing is the highly predictable alteration of light and darkness. Living organisms follow these periodic changes in the environment and display biological variations in 24-h intervals, called daily rhythms. The human sleep-wake cycle constitutes perhaps the best known example of a behavior that occurs with a ~24 h periodicity. Many other organisms display comparable behaviors, commonly referred to as the rest-activity cycle, taking into account that "rest" is not always "sleep". Also many behavioral and physiological processes display daily rhythms, such as locomotor activity, feeding, body temperature, cardiovascular function, and hormonal secretion. The issue is how these rhythms are generated, or even whether they are internally generated or a just a passive consequence of the changes in the environment.

The first observation of rhythmic behavior in human history goes back to the 4th century B.C., when Androsthenes of Thasos, a ship captain under the command of Alexander the Great, recorded his observation of daily movements of plants (Refinetti, 2006). Androsthenes traveled North Africa and India where he observed the daily movements of the tamarind tree (*Tamarindus indica*). Androsthenes noticed the daily cycles of movement of tamarind leaves in which leaves move up during the day and down at night. Further, observations about daily rhythmicity were made by great physician Hippocrates. Hippocrates (460-370 B.C.), who is also considered as the father of medicine, observed periodic physiological processes, such as changes of physiological condition and disease state over 24 h (Refinetti, 2006). These observations point out that daily physiological rhythms may be caused not only by

environmental factors (such as the alternation of day and night), but also generated within the organism in the absence of environmental cycles. The notion of endogenous rhythm came to the scientific community after the publication of a monograph by a French astronomer, Jean-Jacques Dortous de Mairan (1678-1771) in which he mentioned his observation about daily opening and folding of the leaves continued even when the plant was placed in the dark room. This observation suggested that the movements are not the result of a response of sun. Rather it is controlled within the plant. The concept of endogenous rhythms was not accepted despite these findings because of the arguments that all environmental influences that result from the rotation of Earth could not have eliminated in darkness.

The 20th century witnessed a surge in sophisticated research on biological rhythms and thus the establishment of the discipline of chronobiology. In the 1930s Kalmus and Bünning argued for an endogenous timing mechanism based on the finding that periodicity of the rhythm of adult emergence in *Drosophila* varied with temperature fluctuations that were clearly independent of Earth rotation (Sehgal, 2004). The idea that endogenous clocks control the circadian rhythms (rhythms of approximately 24 hours periodicity that persistent in constant environmental condition) gained wider acceptance in the next decades. Interspecies variations in the periodicity of circadian rhythms under non-cycling environmental conditions helped to strengthen this notion. More importantly, the period in most cases did not precisely match that of any environmental cycle, indicating that it was most likely endogenously generated (Sehgal, 2004). For instance, in constant darkness (DD), activity of a rat each day begins and ends a little later than it did the day before. Usually the drift that occurs because of the periodicity of this rhythm, and thereby that of underlying clock, is a little bit more than 24 h. This drift is called "free run". A free-running rhythm of locomotor activity is illustrated below (**Figure 1**). In 1960s Jürgen Aschoff and his colleague Rütger Weber demonstrated that in the absence of environmental cues (light), also the human sleep-wake rhythm persists, and free-runs with a period longer than 24 h (Aschoff, 1965). A similar observation was also made by the French explorer and scientist (Siffre, 1964), when he isolated himself in a deep cave without any time cues for several months. A free-running rhythm, with a cycle deviant from 24 h in constant conditions, is today considered as proof of endogenous generation. The vast majority of living species that evolved on our continuously rotating planet have developed an internal oscillating system to match these external variations. This major conclusion was first drawn in the landmark Cold Spring Harbor Symposium on Biological Clocks in 1960, organized by Jürgen Aschoff and Colin Pittendrigh, an American biologist considered as another co-founder of modern biological rhythm research. Pittendrigh summarized all the

knowledge accumulated by the pioneers of the field and established the basis of chronobiology (Pittendrigh, 1960).
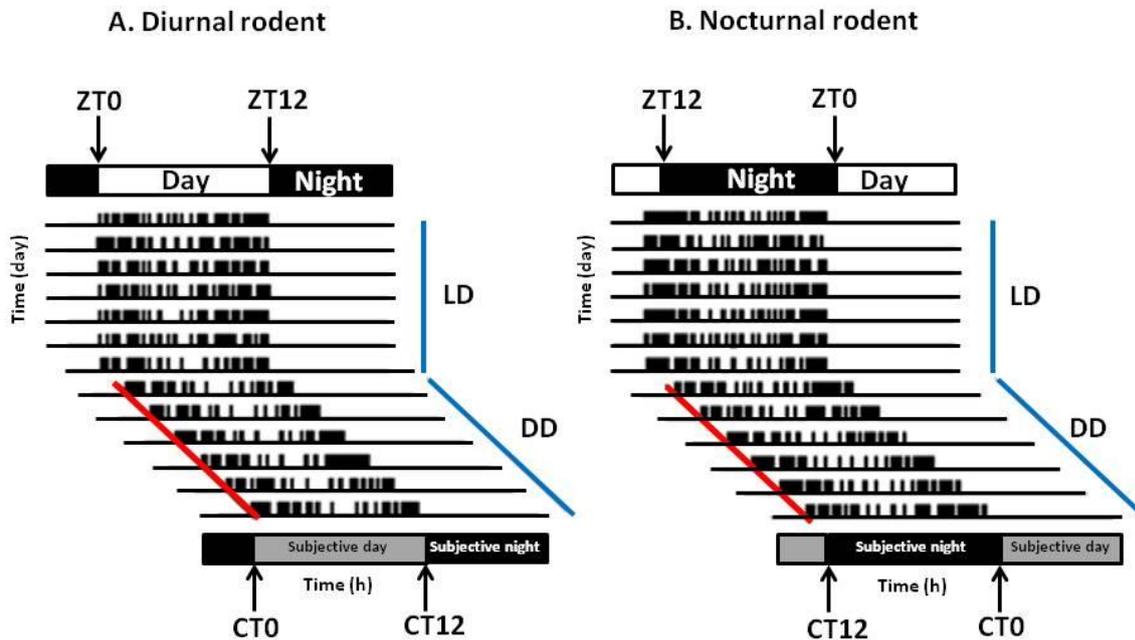

Figure 1. Schematic representation of actograms of a diurnal and a nocturnal rodent
The actograms represent rhythms of locomotor activity initially entrained to a 24-h light/dark (LD) cycle. Each horizontal line corresponds to one day and vertical black data bars represent bouts of activity. Above the actograms, white and black bars indicate the light and dark conditions, respectively. Upon transfer in constant darkness (DD), circadian rhythms resume with their endogenous period (> 24-h here). ZT: *Zeitgeber* Time (ZT0 corresponding to lights on); CT: Circadian Time.

Today we know that almost all the organisms from cyanobacteria to humans show circadian rhythms. For this introduction, my prime focus is to describe the circadian clock system in mammals, its daily entrainment to synchronizing cues, and its control over metabolism and physiology in nocturnal and diurnal species.

3. Circadian clock system

3.1 Circadian rhythm properties

The periodicity of ~24 h is one attribute of the circadian rhythms. A second circadian characteristic is that these rhythms can be synchronized or reset by environmental cues. The dominant environmental signal that affects circadian rhythms is light. In 1954 Jürgen Aschoff used for the first time the German term *Zeitgeber* ("time giver") to define an exogenous (external cue) that triggers some sort of change in an organism's endogenous clock to synchronize or set the phase of circadian rhythms (Aschoff, 1954). The process of synchronization itself is called *entrainment*. In chronobiology, time is defined according to

the environmental cycle and called *Zeitgeber Time* (ZT). For example, in a 24-h cycle that consists of 12 h of light followed by 12 h of darkness, ZT 0 corresponds to "lights on" and ZT 12 is "lights off" (Fig.1). Persistence of periodicity in the absence of a cyclic environment is another property of circadian rhythms. Under these conditions, rhythms are said to be free-running. The endogenous natures of the rhythms and their periodicity have to be determined under free-running conditions. Under constant environmental conditions, the time points are referred to as *Circadian Time* (CT) (**Figure 1**), which is the time generated by the internal clock.

Colin Pittendrigh in the early 1950s demonstrated that the eclosion (i.e., hatching of adult flies from the pupae) rhythm in *Drosophila pseudoobscura* persists in constant conditions and can be entrained by a light cycle close to the flies' endogenous period. More importantly, the period of the eclosion rhythm remains relatively constant when exposed to changes in environmental temperature. In other words, the eclosion rhythms of *Drosophila* were temperature compensated, unlike the rates of most of biochemical reactions (Pittendrigh, 1954). Thus, the circadian period remains constant over a wide temperature range.
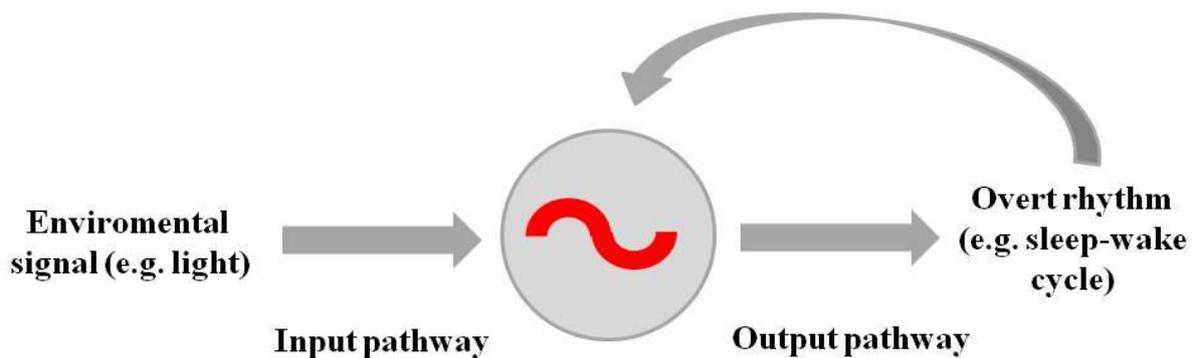
Figure 2. Schematic representation of a simple circadian system

Adapted from Roenneberg and Merrow (2003)

The fundamental properties of the clocks can be represented as a circadian system **(Figure 2)**. There can be multiple input and output pathways to serve a single clock, an output being able to feedback to the clock. This thesis is based on this theoretical framework (See chapter 4).

3.2 The suprachiasmatic nucleus: the biological clock

3.2.1 Localization and properties

The first indication that the medial part of the hypothalamus is involved in daily rhythms of locomotor activity in rats came from the lesion experiments of Richter (1967). A few years later, the effect of removal of the suprachiasmatic nuclei (SCN) by bilateral electrolytic

lesions was tested by Moore & Eichler (Moore and Eichler, 1972) and Stephan & Zucker (Stephan and Zucker, 1972) in rats **(Figure 3)**.

Figure 3. Localization of the suprachiasmatic nucleus (SCN) in golden hamster

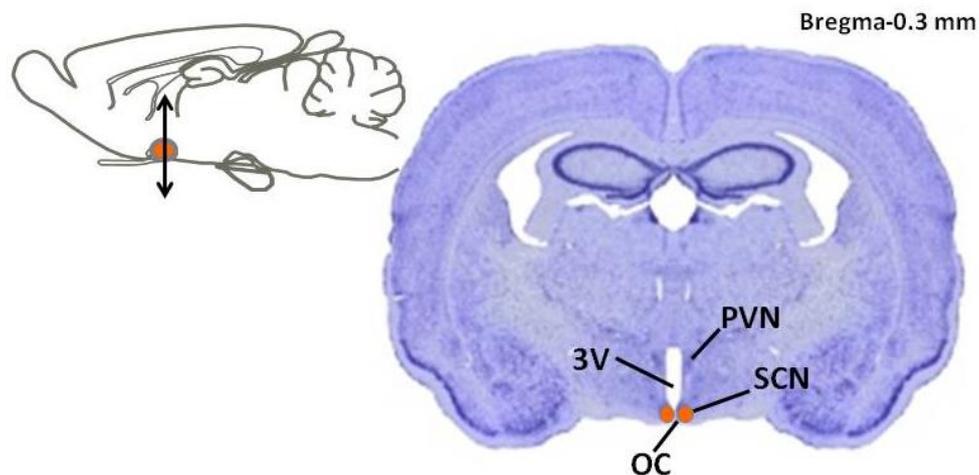3V: third ventricle; OC: optic chiasm; PVN: paraventricular hypothalamic nuclei, SCN: suprachiasmatic nuclei

Adapted from Morin and Wood (2001)

Ablation of SCN abolished the behavioral and hormonal rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972; Moore and Klein, 1974; Ibuka and Kawamura, 1975; Nagai et al., 1978). Later these observations were also confirmed in other mammals (Edgar et al., 1993a; DeCoursey et al., 1997) **(Figure 4)**.
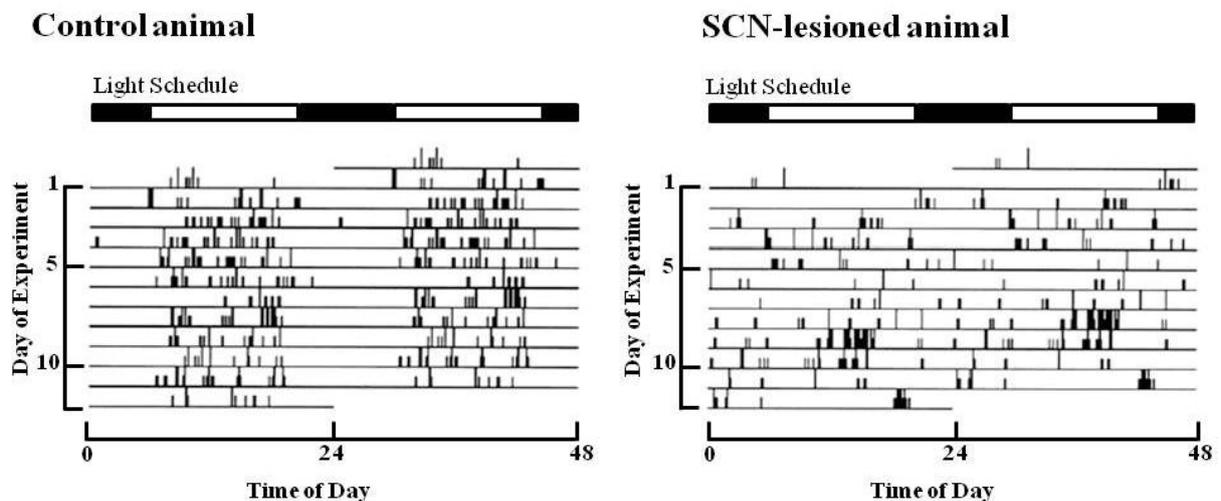
Figure 4. Effect of SCN lesion in the diurnal rodent, *Ammospermophilus leucurus*

Two actograms representing the locomotor activity of an intact SCN animal (left) and a SCN-lesioned animal (right). Each horizontal line corresponds to one day and vertical black data bars represent bouts of activity. Above the actograms, white and black bars indicate the light and dark conditions, respectively.

Adapted from DeCoursey et al., (1997)

Electrical recordings showed that the SCN is an endogenous pacemaker, as SCN neurons display circadian patterns of firing with highest activity during subjective daytime even when isolated *in vivo* (Inouye and Kawamura, 1979) or maintained as cell culture *in vitro* (Bos and Mirmiran, 1990; Welsh et al., 1995). Another approach, using ^{14}C labeled deoxyglucose,

corroborated that the clock is an endogenous oscillator by demonstrating a rhythmic pattern in glucose utilization in the SCN which also persisted in constant darkness (Schwartz and Gainer, 1977; Schwartz et al., 1987). The final evidence that the SCN is "the master clock" came from graft experiments of Ralph and colleagues (Ralph et al., 1990). They identified a mutant hamster characterized by a shorter endogenous period length, called Tau mutant. By performing lesions in the SCN (SCN-X) of Tau mutant and wild-type hamsters, they obtained arrhythmic animals. When they transplanted SCN grafts of Tau mutant in SCN-X wild-type animals, they restored rhythmicity with a period characteristic of the Tau mutant, and *vice versa*. In other words, rescued rhythms always exhibited the period of the donor genotype. All these findings demonstrated that the SCN is the main conductor of circadian rhythmicity in mammals.

The studies of single cell electrical activity from SCN slice preparations or dissociated cell cultures from the rodent brain suggest that even individual cells exhibit circadian rhythmicity, that different cells can have different circadian periods, and that the overall period of the circadian oscillation generated by the SCN is the average of the periods of the individual cells (Welsh et al., 1995; Liu et al., 1997; Honma et al., 1998; Honma et al., 2004). The dissociated neurons oscillating with different periods and phases become mutually synchronized by coupling and thereby are able to produce a coherent SCN output. Thus, SCN neurons are relatively unstable oscillators which require network interactions to stabilize their noisy cycling. As a tissue, the SCN thus provides a more precise and robust output than single SCN neurons (Herzog et al., 2004; Liu et al., 2007). Attempts to identify the coupling mechanism suggest that neuronal firing and synaptic communication between neurons are required (Welsh et al., 2010).

3.2.2 Neurotransmitters of SCN

The SCN is a heterogenous structure based on its population of neuronal and glial cell types. Left and right SCN each contain around 10,000 neurons in two anatomical subdivisions: the ventrolateral (core) region, receiving retinal input, and the dorsomedial (shell) region receiving input from the core (Abrahamson and Moore, 2001) (**Figure 5**). Much is known about the neurotransmitters/neuropeptides used by the SCN neurons: γ amino butyric-acid (GABA, major inhibitory neurotransmitter in the central nervous system), Arginine Vasopressin (VP or AVP) and Vasoactive Intestinal Polypeptide (VIP), Gastrin Releasing Peptide (GRP), cholecystokinin (CCK), Substance P (SP) and somatostatin are among the predominant ones. GABA seems to be the most common neurotransmitter of the SCN (Moore

and Speh, 1993). Most neuropeptides co-localize with GABA, and many synapses between SCN neurons are GABAergic (Moore and Speh, 1993; Buijs et al., 1995; Strecker et al., 1997), though few SCN neurons are glutamatergic (Cui et al., 2001).
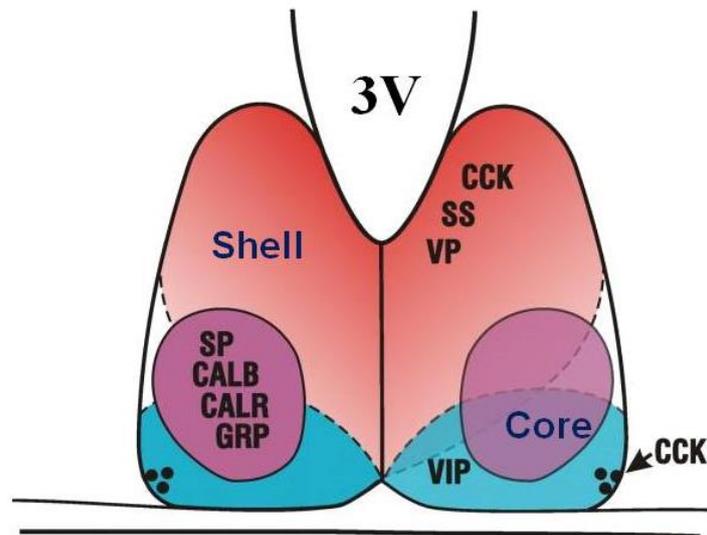

Figure 5. Schematic representation of neuropeptides distribution in SCN

Many of the neurons within the core SCN express VIP, GRP and CALB. In the shell region SCN neurons mostly express VP. Most of these neurons also express GABA and very few glutamates (not shown here). 3V: third ventricle; VP: vasopressin; SS: somatostatin; CCK: cholecystokinin; SP: substance P; CALB: calbindin; CALR: calretinin; GRP: gastrin releasing peptide; VIP: vasoactive intestinal polypeptide.

Adapted from Morin (2013)

AVP is also abundant in the SCN, predominantly in the dorsomedial region of the nucleus (Ibata et al., 1999; Moore et al., 2002; Morin et al., 2006; Nascimento et al., 2010). AVP expression is higher during the day than during the night in rodents (both nocturnal and diurnal) and humans maintained under light-dark cycles (Tominaga et al., 1992; Hofman and Swaab, 1993; Dardente et al., 2004). The rhythmic expression of *Avp* persists in constant darkness (Dardente et al., 2004). Other studies show that AVP is also rhythmically released from the synaptic terminals with a higher rate during the day (Kalsbeek et al., 1998). In contrast to AVP, VIP is present mostly in the ventrolateral region of the SCN and in its dorsal projections. Ventrolateral neurons of the SCN also express other neuropeptides, like calbindin, calretinin, SP, neurotensin and GRP. VIP release is not consistent across the species, in Syrian hamster it has been shown that VIP release is highest in mid-day whereas in Golden hamster and humans lowest expression of VIP in SCN were reported in day time (Refinetti, 2006). On the other hand, GRP content in SCN increases over the course of the light period and gradually decreases during the dark period in the rat (Shinohara et al., 1993). Neurotransmitters involved in SCN inputs and outputs are discussed in subsections 3.3 and 3.4 of this Introduction.

3.2.3 Mammalian Molecular Clock

The clock mechanism in the SCN involves 24-h oscillations of core clock components, called clock genes and defined as genes whose protein products are necessary for generation and regulation of circadian rhythms within individual cells (Ko and Takahashi, 2006). The molecular oscillations of the SCN depend on several clock genes. Circadian rhythmicity is based on rhythmic expression of core clock genes and their autoregulatory feedback transcriptional/translational loops (**Figure 6**). The core clock machinery is structured with positive transcriptional regulators, including BMAL1/CLOCK and RORs, and negative regulators such as PERs (PER1-3), CRYs (CRY1-2) and REV-ERBs. BMAL1 binds with CLOCK and forms heterodimers which activate the transcription of the negative PER and CRY regulators, thus defining a positive loop (Reppert and Weaver, 2002). PERs and CRYs accumulate in the cytoplasm and form complexes which translocate into the nucleus to inhibit their own transcription along with other BMAL1/CLOCK-driven transcription, such as that of the clock-controlled genes, thereby forming a negative loop (Mohawk et al., 2012). An additional negative loop in this molecular network is contributed by the nuclear receptors REB-ERBα and β. REV-ERBs bind to the ROR response element (RRE) of BMAL1 and CLOCK promoters and repress their transcription, whereas in counterbalance to REV-ERBs inhibition, RORα/β/γ also bind on the RRE of BMAL1 and induce its transcription (Preitner et al., 2002; Guillaumond et al., 2005; Crumbley and Burris, 2011).

The circadian rhythms generated by this molecular clock machinery get fine-tuned by environmental cues. In particular, light can synchronize the SCN neurons via a cascade of transcriptional activation. In response to photic inputs from retinal ganglion cells, glutamate and pituitary cyclase-activating peptide (PACAP) are released in the ventral region of the SCN, leading to transcriptional induction of clock gene (*Per1* and *Per2*) expression through chromatin remodeling (Dibner et al., 2010) (a more detailed description follows in section 3.3.1). Of note, the molecular clock in the SCN works at the same astronomical times in diurnal and nocturnal species, and the mechanisms underlying photic resetting are essentially similar in terms of temporal sensitivity and direction of light-induced phase shifts between both categories of mammals (Challet, 2007). These findings suggest that the distinction between nocturnal and diurnal animals likely relies on neural mechanisms operating downstream of the SCN clock (Kalsbeek et al., 2008b).

Figure 6. The molecular mechanism of the mammalian circadian clock in the SCN and other tissues
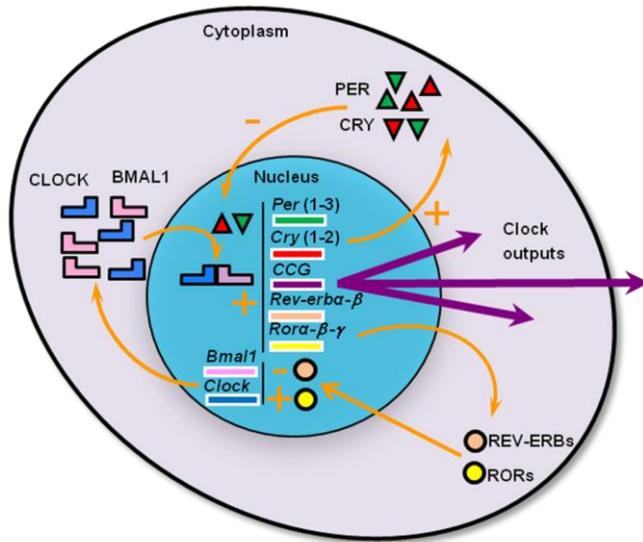
The core circadian clock is formed by the positive and negative limbs of a transcriptional-translational feedback loop. In the positive limb, BMAL1 and CLOCK form heterodimers and activate the transcription of *Per* (1-3) and *Cry* (1-2). PER and CRY proteins repress their own transcription by inhibiting CLOCK-BMAL1 activity in the negative limb. In an additional loop, REV-ERBs repress *Clock* transcription, whereas opposite actions of REV-ERBs and RORs contribute to the rhythmic expression of *Bmal1*. In addition, the CLOCK-BMAL1 heterodimer also activates transcription of clock controlled genes, including many metabolic genes as clock outputs. BMAL1, brain and muscle ARNT-like 1; CLOCK, circadian locomotor output cycles kaput; PER, Period; CRY, Cryptochrome; REV-ERBs, reverse viral erythroblastosis oncogene products; RORs, retinoic acid-related orphan receptors; CCG, Clock controlled genes.
Adapted from (Crumbley and Burris, 2011; Mohawk et al., 2012).

Demonstration of rhythmic clock gene expression in cells and tissues throughout the body, and persistence of these rhythms in cultured cells revealed the presence of the molecular clock mechanism also in brain regions outside of the SCN, such as the arcuate nucleus (ARC) and the dorsomedial hypothalamic nuclei (DMH), as well as in most peripheral organs, including those essential for energy homeostasis, such as the liver, kidney, pancreas, skeletal muscle and adipose tissues (Balsalobre et al., 1998; Yamazaki et al., 2000; Abe et al., 2002; Yoo et al., 2004; Guilding and Piggins, 2007). Although the basic molecular components of the central clock are conserved in these extra-SCN oscillators, their self-sustained rhythmicity is less robust than in the central clock. Using *Per1* luciferase rat explants, Yamazaki et al. (2000) showed that circadian rhythmicity in peripheral organs, such as lungs, liver and skeletal muscle, starts to dampen within one week, whereas the SCN cycle up to at least 32 days. Dampening within a few cycles has also been reported for explants of various brain areas other than the SCN (Abe et al., 2002; Abraham et al., 2005; Guilding and Piggins, 2007). Of note, Yoo et al. reported persistent, self-sustained circadian oscillations of PER2 expression for more than 20 days in lungs and liver explants of PER2-luciferase transgenic mice (Yoo et al., 2004). SCN lesions in this study do not cause disappearance of PER2 rhythmic expression, although they do lead to asynchrony of phase between peripheral organs of the same individuals which specifies the role of the SCN as a conductor which drives rhythms of peripheral clocks by maintaining phase coherence among organs.

3.3 Circadian entrainment

A circadian clock maintains a self-sustained rhythm even in the absence of environmental cycles, thus ensuring that internal functions maintain their temporal relationships under constant conditions. However, the real life usually gives temporal cues that help rhythms to adapt and anticipate to natural periodic changes, including light/dark, temperature, humidity, social activity and food availability. These environmental cues are broadly classified in photic and non-photic cues. The way by which endogenous rhythms can be entrained by light and darkness became the focus of many studies around the 1960s. The investigation of the entrainment process by the non-photic cues began later in the late seventies.

3.3.1 Photic entrainment

Light is the most ubiquitous entraining signal or *Zeitgeber* encountered in the daily life of most of organisms. As the SCN-driven period of circadian rhythms differs slightly from 24-h in most mammals, rhythms must periodically be shifted forward or backwards in order to maintain an appropriate phase-relationship with the environmental period of exact 24-h (Challet, 2007). Brief pulses of light delivered to animals housed in constant darkness produce phase-dependent shifts in the timing of the rest-activity cycles, thereby defining a so-called phase-response curve (PRC) (Daan and Pittendrigh, 1976; Schwartz and Zimmerman, 1990) **(Figure 7)**. Exposure to light during the early night, for instance, when nocturnal animals have just become active after dusk, delays the clock. This shift will delay activity onset to a slightly later time on subsequent cycles, realigning with the external environment. Conversely, light encountered during the late night, or when a nocturnal rodent remains active after dawn, advances the phase of clock. This shift will advance activity onset to a slightly earlier time on subsequent days, again realigning the behavior and physiology with the external world. As illustrated in **Figure 7** for nocturnal rodents, light in early and late subjective night produces phase-delays and phase-advances, respectively, whereas light during most of the subjective day has no phase-shifting effect. These circadian responses to light are roughly similar in both nocturnal and diurnal species (Slotten et al., 2005; Challet, 2007).
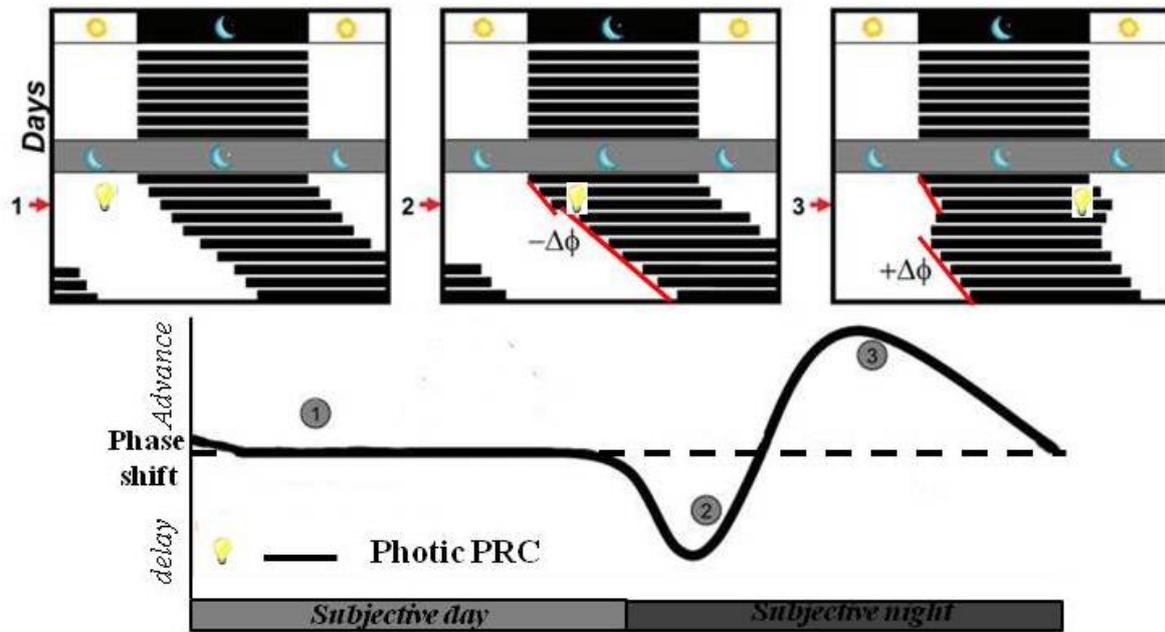
Figure 7. Phase response curve (PRC) of nocturnal animals to light pulses

The free-running locomotor activity rhythm of animals transferred to constant darkness can be phase-shifted in a phase dependent manner. Phase advances are plotted as a positive value ($+\Delta\phi$) and phase delays as negative values ($-\Delta\phi$). The photic PRC consists of three characteristic zones (1) the dead zone: a light pulse during most of the subjective day does not cause a phase shift; (2) the delay zone: at the beginning of the subjective night and (3) the advance zone: toward the end of the subjective night.

Adapted from Golombek and Rosenstein (2010)

Phase-shifts such as those described above in response to light pulses are generally evaluated by measurements of daily onsets or offsets of the locomotor activity pattern. Of note, these shifts in behavior actually correspond to phase-changes of the underlying SCN clock. Now the question remains: how does light reset the SCN clock? In 1972 (Moore and Lenn) discovered terminals of retinal axons in the SCN, strongly suggesting that the mammalian visual system could convey photic information to the SCN (Moore and Lenn, 1972). Photic signals from the retina are conveyed to the SCN directly via a neural pathway called the retinohypothalamic tract (RHT). Additionally, an indirect neural pathway courses to the SCN via the intergeniculate leaflet (IGL) **(Figure 8)**.

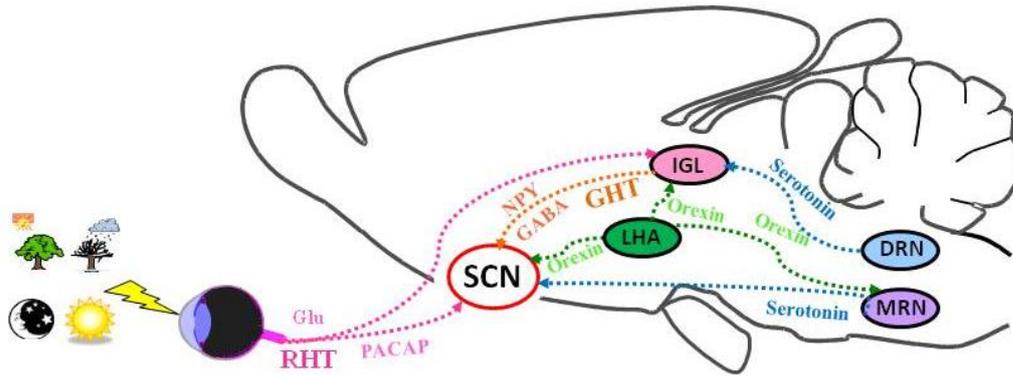**Figure 8. Schematic of a sagittal brain section showing nuclei and neurotransmitters of the mammalian circadian system involved in photic and non-photic resetting.**

Photic signals are transmitted directly to the SCN via the RHT utilizing Glu and PACAP. Non-photic information reaches the SCN via a network of connections involving the NDR and NMR, the IGL and the LHA. These pathways use serotonin, NPY, GABA, and orexin respectively as their main transmitters. NDR: dorsal raphe nucleus; GABA: γ-amino butyric acid; GHT: geniculohypothalamic tract; Glu: glutamate; IGL: intergeniculate leaflet; LHA: lateral hypothalamic area; NMR: median raphe nucleus; PACAP: pituitary adenylate-cyclase-activating peptide; NPY: Neuropeptide Y; RHT: retinohypothalamic tract; SCN: suprachiasmatic nucleus.

Adapted from Hughes and Piggins (2012)

Still, the big question is which retinal cells, and which molecules in those cells, act to detect light and to convey this information to the SCN clock? And so the search for the "circadian photoreceptor", responsible for photic resetting of the circadian clock and localized within the eye, began. Freedman et al. (1999) observed that mice lacking both rod and cone photoreceptors show normal resetting of their activity rhythm by light. This observation led to the idea of a non-visual circadian photoreceptor (Freedman et al., 1999). Meanwhile, a group of retinal ganglion cells were shown to contain a light sensitive photopigment, called melanopsin, that was highly responsive to blue-light stimulation (Provencio et al., 2000). Mice lacking the melanopsin gene displayed severely attenuated phase-resetting in response to brief pulses of monochromatic light, demonstrating the critical role of melanopsin in circadian photo-entrainment. The axons of these retinal ganglion cells form the RHT and project monosynaptically to the ventral (core) of the SCN (Hattar et al., 2002), where they release mainly glutamate and PACAP (Castel et al., 1993; Hannibal et al., 1997).

As mentioned above, the RTH projects not only to the SCN, but also to the IGL, a distinct subdivision of the lateral geniculate complex in the thalamus (Pickard, 1985; Moore and Card, 1994; Hattar et al., 2006). From the IGL, the geniculohypothalamic tract (GHT) projects to the SCN and thus indirectly conveys light information by releasing neuropeptide Y (NPY) and GABA. The delay between RHT and GHT signals may provide additional information leading to a more differentiated response of the SCN to light cues. Finally, other structures

could also convey indirect light information to the SCN. For example, the lateral hypothalamus (LH) is also a target of the RHT (Hattar et al., 2006) and the arousal-promoting orexin neurons in the LH project to the immediate vicinity of the SCN (McGranaghan and Piggins, 2001; Brown et al., 2008).

The process of photic entrainment depends upon the direct and indirect transmission of light information to the SCN. The downstream signaling of this transmission induces acute expression of several immediate early genes, such as *c-fos,* and the clock genes *Per1* and *Per2* (Albrecht et al., 1997; Shigeyoshi et al., 1997). In response to light exposure, glutamate and PACAP are released from the RHT and bind to their receptors expressed in ventral SCN neurons (Reppert and Weaver, 2002). More precisely, SCN neurons express several subtypes of glutamate (NMDA-R, AMPA-R) and PACAP (PAC1-R) receptors. Stimulation of these receptors induces Ca^{2+} influx which activates several kinase pathways (Morse and Sassone-Corsi, 2002; Hannibal, 2006). One of these pathways involves the extracellular signal-regulated kinase (ERK), which belongs to the family of Mitogen-activated protein kinase (MAPK), known to phosphorylate the cAMP response element-binding protein (CREB). Many pathways converge at phosphorylation of CREB and stimulate the transcription of *Per1* and *Per2* genes by its binding to a CRE element in the promoter region (Travnickova-Bendova et al., 2002; Tischkau et al., 2003). Light responses of the clock are complex because there are many pathways at work. Up to this stage, such intracellular signaling cannot explain explicitly the differential effects of light inducing phase-delays and advances, according to circadian time of the light pulse. Clearly the molecular mechanism of photic entrainment is not fully understood yet (Sehgal, 2004) **(Figure 9)**.
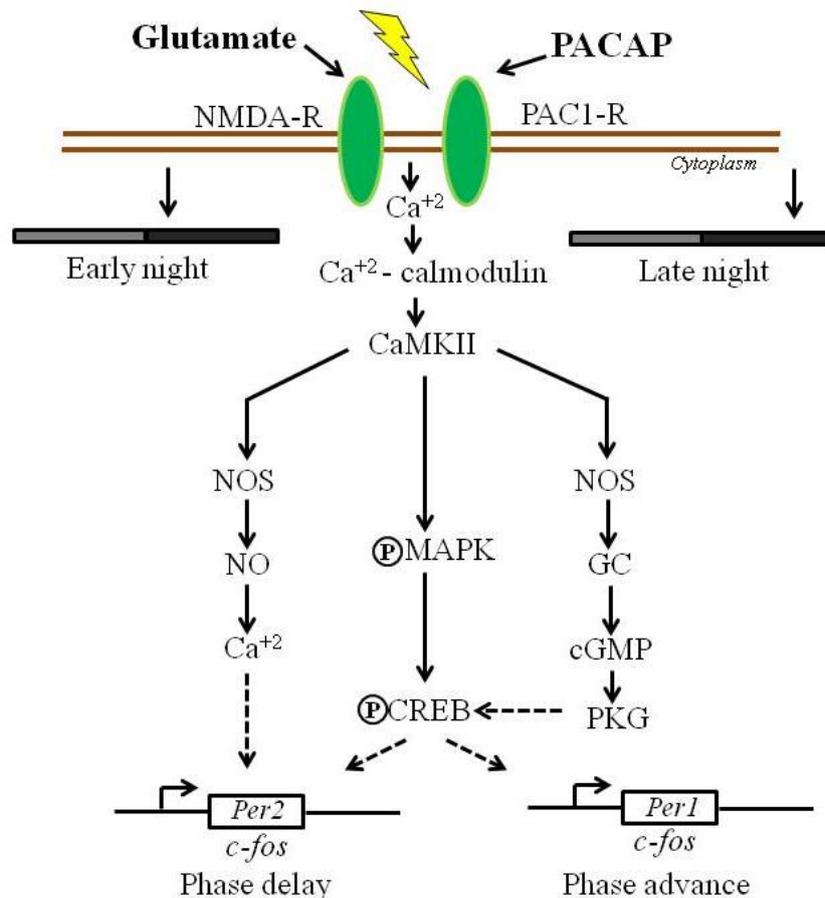
Figure 9. Molecular pathways for phase delays and phase advances in the SCN

Photic stimulation triggers release of glutamate and PACAP, which than interact with their receptors at the membrane of ventral SCN cells. The resulting influx of Ca+2 stimulates CaMKII, which phosphorylates NOS. Molecular cascades downstream from CaMKII/NOS are not completely clear for differences between phase delays and advances. The phosphorylation of CREB mediated by MAPK and activation of PKG by cGMP could be the convergent point for both delays and advances. However Ca^{2+} release from the endoplasmic reticulum through ryanodine receptor activation seems to be involved in phase delays. This is a simplified view of the photic signaling cascade without considering the discrepancies in the literature. NMDA-R: N-methyl–D-aspartate receptor; Ca^{+2}: Calcium; CaMKII: Calcium Calmodulin Kinase II; NOS: Nitric oxide synthase; NO: Nitric oxide; cGMP: cyclic Guaosine monophosphate; GC: guanylyl cyclase; PKG: cGMP-dependent protein kinase; P-MAPK: Phosphorylated Mitogen-activated protein kinase; P-CREB: Phosphorylated cAMP response element binding protein; Per: Period.

A*dapted from (Sehgal, 2004; Hannibal, 2006; Golombek and Rosenstein, 2010)*

Moreover, the effect of light is not only limited to transcriptional changes, such as *c-Fos* and *Per* expression, but it also impacts on post-transcriptional modifications of core clock components. For example, light-induced degradation of BMAL1 could constitute an important step for entrainment (Tamaru et al., 2000). The intricacy of photic entrainment is getting even more intensified by epigenetic interventions, such as light-induced chromatic remodeling (Crosio et al., 2000).

3.3.2 Non-photic entrainment

The concept that circadian rhythms could be influenced by mechanisms other than light came to prominence through a series of experiments demonstrating that the rhythms of birds could be entrained to the daily playback of birds songs (Gwinner, 1966; Menaker and Eskin, 1966). The early reports of social entrainment in humans (Aschoff, 1979; Weaver, 1979) also revealed that circadian rhythms could be altered using so-called non-photic stimuli independent of the light itself or the specific neural pathways activated by light. A wide variety of different stimuli fall into this category, such as: temperature, food availability, social interaction and behavior arousal. In late 1980s, Mrosovsky and Salmon demonstrated that enhanced locomotor activity acts as potent zietgeber in hamsters (Mrosovsky and Salmon, 1987, 1990). Furthermore, it has been shown that enhanced physical activity is able to alter the period (Yamada et al., 1986) or produce phase-shifts (Reebs and Mrosovsky, 1989a) of circadian rhythms in nocturnal rodents. Physical activity can also shift the human circadian rhythms. Notably, exercise during early evening and after midnight elicits phase advances and delays, respectively (Van Reeth et al., 1994; Buxton et al., 1997; Buxton et al., 2003). The PRC obtained with non-photic cues is called the "non-photic PRC". The non-photic PRC of nocturnal rodents is characterized by phase-advances during the subjective day and little or no response during the subjective night (**Figure 10**).

Unlike the light PRC, whose global shape is almost similar for nocturnal and diurnal species and extensively researched, the non-photic PRC for diurnal rodents has not been extensively studied. Entrainment to scheduled locomotor activity has been reported in diurnal rodents and primates (Hut et al., 1999; Glass et al., 2001), as well as weak locomotor activity feedback effects in the diurnal/crepuscular Degus (Kas and Edgar, 1999). The characteristics of non-photic entrainment in these diurnal species suggest that the diurnal non-photic PRC may be close to that of nocturnal species. However, results from dark pulse experiments suggest differential responses of non-photic stimuli between nocturnal and diurnal species (Challet, 2007). To understand how behavioral arousal acts as a non-photic cue in the diurnal rodent Grass rat, *Arvicanthis ansorgei* is one of goal of this thesis.
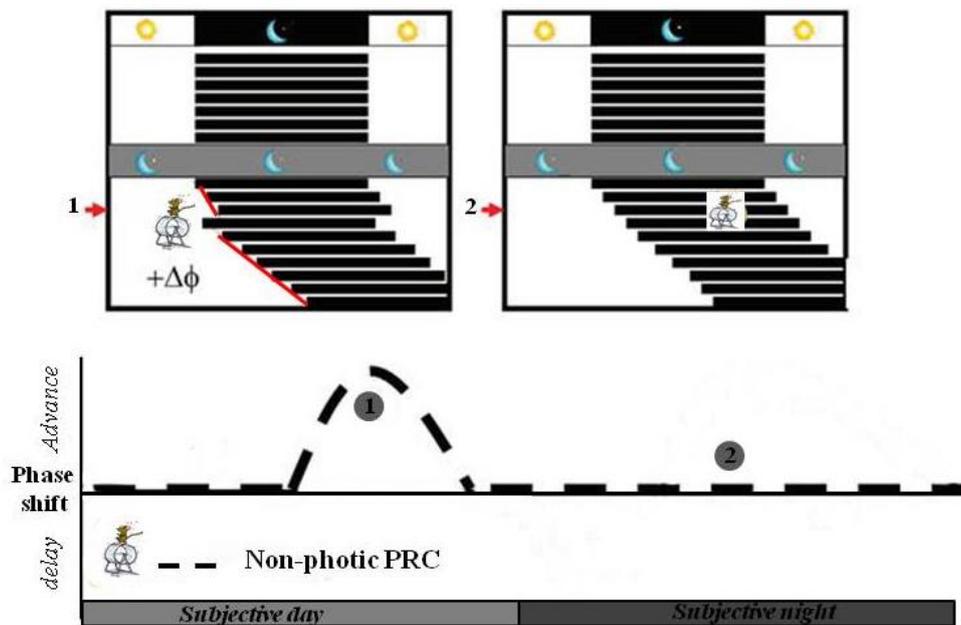
Figure 10. Phase response curve (PRC) to a non-photic stimulus, such as a novel running wheel, in the nocturnal hamster

The free-running locomotor activity rhythm of a nocturnal animal transferred to constant darkness (1) can be phase advanced by arousal in the subjective day, whereas (2) little or no effect on the behavioral phase occurs when aroused in subjective night.

Adapted from Golombek and Rosenstein (2010)

At the molecular level, the *Per* genes are the main targets within the core molecular oscillator for stimuli that reset the clock. Non-photic phase-shifts induced by increased locomotor activity produce a down-regulation of the expression of *Per1* and *Per2* mRNA in the SCN (Maywood et al., 1999; Yokota et al., 2000). Furthermore, injections of *Per1* antisense oligonucleotides in the mid-subjective day (i.e., the approximate time of maximal non-photic phase shifts and maximal *Per1* expression) suppress *Per1* levels and induce non-photic-like phase-advances (Hamada et al., 2004). The molecular mechanisms of signal transduction that mediates the suppressive action of non-photic cues on SCN *Per* gene expression are poorly understood. Non-photic stimuli may suppress ERK phosphorylation in the SCN (Coogan and Piggins, 2005). Moreover, treatment with serotonin agonists during the subjective day, produces phase-advances and markedly suppresses *Per1* and *Per2* mRNA levels (Horikawa et al., 2000; Duncan et al., 2005).

The two major input pathways that are considered to transmit non-photic information to the SCN are the GHT from the IGL, that also conveys photic information to SCN (Harrington, 1997), and serotoninergic input from the median raphe nuclei in the brainstem. The IGL also receives a serotonergic projection from the dorsal raphe (Hughes and Piggins, 2012) (**Figure**

8). As mention above, the IGL releases NPY and GABA. There is evidence that IGL and NPY transfer non-photic signals to the central circadian system. It has been shown that lesions of IGL attenuate the phase-shifts induced by enhanced physical activity in mice and hamsters (Johnson et al., 1988; Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Koletar et al., 2011). *In vivo* electrical stimulation of IGL and NPY infusion in the third ventricle both result in behavioral phase-shifts that resemble the traditional non-photic PRC seen in response to induced locomotor activity (Albers and Ferris, 1984; Rusak et al., 1989; Biello and Mrosovsky, 1996). Infusion of NPY into the region of SCN *in vivo* and directly on the SCN in slices *in vitro* suppresses *Per1* and *Per2* expression (Fukuhara et al., 2001; Maywood et al., 2002). Furthermore, circadian variation of serotonin content in the SCN correlates with physical activities at different times of the day (Shioiri et al., 1991). Electrical stimulation of the raphe nuclei leads to behavioral phase-shifts with increased levels of serotonin in the SCN (Dudley et al., 1999; Meyer-Bernstein and Morin, 1999).

Feeding and metabolic cues are other non-photic signals that can entrain the SCN clock. The pathways for these signals comprise nuclei in the mediobasal hypothalamus, such as the arcuate nucleus and the ventromedial hypothalamic nucleus (Challet et al., 1997; Yi et al., 2006), which could integrate metabolic information and energy status before projecting to the SCN.

3.4 SCN outputs

It is important for a pacemaker to have output pathways by which it controls the timing of various targets in the rest of the body. The SCN conveys its circadian signals by neuronal connections, primarily to other hypothalamic sites, and diffusible substances. The structrucal details of SCN efferents have been studied by the various tracing techniques (Watts and Swanson, 1987; Watts et al., 1987; Kalsbeek et al., 1993). The SCN projects mainly to three areas: hypothalamus, thalamus and septum (**Figure 11**). The SCN core projects to shell and to other hypothalamic areas in its vicinity, particularly the lateral subparaventricular zone (lSPV) (Leak et al., 1999; Leak and Moore, 2001). In general, the dorsomedial SCN which is predominantly containing AVP expressing cells, projects more widely to various areas of the brain, particularly the paraventricular nucleus of thalamus (PVT), paraventricular nucleus of the hypothalamus (PVN), medial subparaventricular zone (mSPV), preoptic area (POA) and dorsomedial hypothalamic nucleus (DMH). Though few VIP fibers can be also found in these areas, there is clear proof for a difference between AVP and VIP targets. The SCN has separate projections to the pre-autonomic sympathetic and parasympathetic neurons in the

PVN (Buijs et al., 2003b). Projections from the SCN proceed monosynaptically to the PVN. Then, the PVN innervates either the dorsal motor nucleus of the vagus nerve (DMV), which contains the parasympathetic motorneurons, or the intermediolateral column of the spinal cord (IML), which contains the sympathetic motorneurons. Via a multi-synaptic pathway SCN efferents target various peripheral organs such as the liver, heart, pineal gland and thyroid gland (Teclemariam-Mesbah et al., 1999; Kalsbeek et al., 2000; Scheer et al., 2001). The brain areas responsible for sleep and wakefulness, such as ventrolateral preoptic area (VLPO) and the nucleus of the locus coeruleus (LC) are also target of SCN efferences (Aston-Jones et al., 2001; Mistlberger, 2005). Ventrolateral preoptic nucleus (VLPO) and median preoptic nucleus (MnPO) possess the sleep active cells, and are scattered through the medial preoptic area (MPOA). The SCN projects only sparsely to these areas, but has a strong indirect projection via the subparaventricular zone (sPVZ). The VLPO, MPOA, and MnPO are mutually connected.
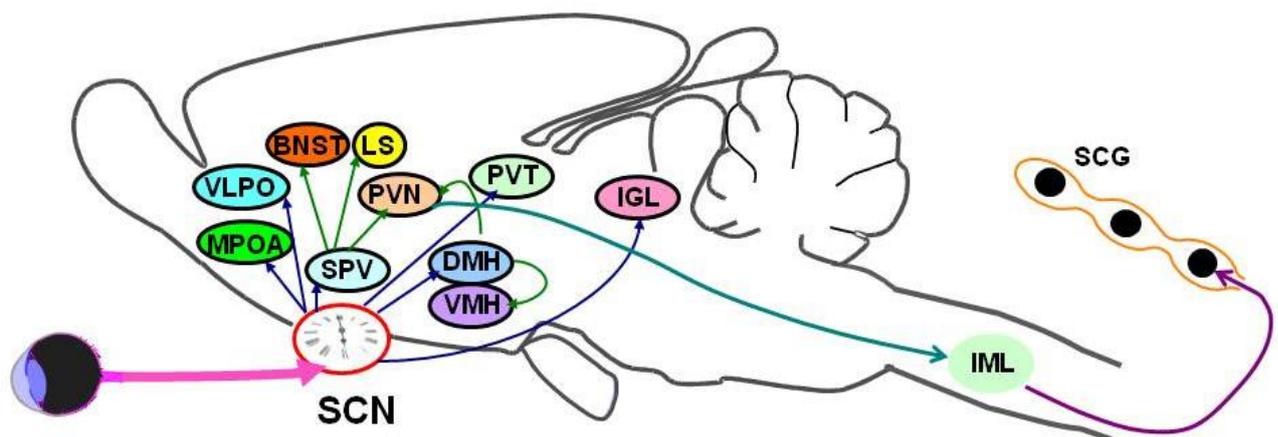
Figure 11. The major efferents of the SCN.

MPOA: medial preoptic area; VLPO: ventrolateral preoptic nucleus; BNST: bed nucleus of the stria terminalis; SPV: subparaventricular area; PVN: paraventricular hypothalamic nucleus; LS: lateral septum; VMH: ventromedial hypothalamic nucleus; DMH: dorsomedial hypothalamic nucleus; PVT: thalamic paraventricular nucleus; IGL: intergeniculate leaflet; IML: intermediolateral column of the spinal cord, SCG: superior cervical sympathetic ganglion

SCN efferents indirectly regulate crucial neuroendocrine and physiological rhythms, such as those of plasma melatonin, glucocorticoid, and glucose concentrations. These rhythms are controlled by a balance of glutamatergic and GABAergic inputs from the SCN to the PVN or the subPVN region (See in section 5.2.2, 5.2.3, 5.3.1). The daily rhythms in body temperature and cardiovascular activity are also under SCN control and clearly dependent on the autonomic nervous system (Scheer et al., 2003). Moreover, other SCN targets such as DMH and MPOA are dependent on the GABA/glutamate balance (De Novellis et al., 1995; Chen et

al., 2003). In addition, the sleep/wake regulatory system in the VLPO seems to depend on the balance of GABA/glutamate input from the SCN as well (Sun et al., 2001).

GABA, glutamate, AVP, VIP, transforming growth factor α (TGF α), prokinectin 2 (PK2), cardiotrophin-like cytokine (CLC), are all strong timekeeping signaling candidates that travel from the SCN to the rest of the brain (Guilding and Piggins, 2007). For example, rhythmic immunoreactivity of AVP has been demonstrated in efferent projections of the SCN (van Esseveldt et al., 2000). Interestingly, as demonstrated by SCN grafts experiments, the humoral release of AVP is important for the restoration of circadian electrical activity in the PVN in the absence of direct neural connections (Tousson and Meissl, 2004). Furthermore, SCN terminal release of AVP has been shown crucial for daily variations in corticosterone levels (Kalsbeek et al., 1992; Kalsbeek et al., 1996). Another candidate, PK2 shows a circadian expression that is modulated by light exposure (Cheng et al., 2005). ICV injections of PK2 inhibit locomotor activity (Cheng et al., 2002). The study in mice lacking PK2 showed its role in the circadian control of locomotor activity (Li et al., 2006). Intriguingly, transplantation experiments of isolated SCN tissue have demonstrated that SCN projections are not required for the control of circadian locomotor activity rhythm, suggesting that diffusible factors such as PK2 may sustain circadian rhythms of behavioral activity (Silver et al., 1996). However, graft transplantation of SCN failed to restore endocrine rhythms (Meyer-Bernstein et al., 1999), showing that the daily SCN output signaling is either accomplished through synaptic connections and/or relies on nearby tissue that are direct targets of local diffusible factors. de la Iglesia et al (1995) have very nicely shown, using split hamsters, that direct SCN projections to the GnRH neurons are necessary for the circadian control of the LH surge.

4. Feedback action of arousal on clock

Arousal is a non-photic cue that has the ability to shift circadian rhythms, alter circadian period and modulate the phase of entrainment to LD cycle. The first evidence of behavioral arousal as a synchronizing factor came from experiments with dark pulses in the nocturnal insectivorous bat, *Taphozous melanopogon* (Subbaraj and Chandrashekaran, 1978). Exposure to dark pulses in the resting phase resulted in phase-advances, while dark pulses in their active phase resulted in phase-delays in bats housed in constant dim light. As in bats, dark pulses in nocturnal rodents produced large advances in the mid-to-late subjective day and smaller phase-delays in late subjective night (Challet, 2007; Webb et al., 2014). These variations in the direction and magnitude of the dark-pulse phase-shifts were close to a non-photic PRC (see section 3.3.2 **Figure 10**). From the dark puls experiments, it is clear that clock resetting

occurs during rest period, irrespective of their locomotor activity pattern in nocturnal and diurnal species (Lee and Labyak, 1997; Mendoza et al., 2007). These findings suggest that circadian sensitivity to dark exposure differs greatly between nocturnal and diurnal species. Since darkness induces hyperactivity and/or arousal in nocturnal animals, it has been proposed that hyperactivity mediates part of the resetting properties of dark exposure (Reebs et al., 1989; Canal and Piggins, 2006). Results from other procedures that stimulate arousal during the usual daily resting period, such as induced locomotor activity or sleep deprivation, produce phase-shifts comparable to those induced by dark pulses in nocturnal rodents (Reebs and Mrosovsky, 1989a; Antle and Mistlberger, 2000). By contrast, some other procedures leading to arousal, such as caffeine and modafinil treatment, remain ineffective to induce behavioral phase-shifts in constant conditions (Webb et al., 2006; Vivanco et al., 2013). Moreover, cues associated with behavioural activation, such as involuntary physical activity, sleep deprivation, activation of adenosine receptors, all decrease photic responses of the SCN clock in nocturnal species (Watanabe et al., 1996; Mistlberger et al., 1997; Challet et al., 2001; Elliott et al., 2001; Sigworth and Rea, 2003).

4.1 Arousal dependent on locomotor activity

A number of methods were used to study the enhanced locomotor activity and their effect on circadian functions which includes confinement to a novel-wheel, injection of benzodiazepine that triggers locomotor activity, and forced treadmill running (Turek and Losee-Olson, 1986; Mrosovsky and Salmon, 1987; Mistlberger, 1991). It has been shown that single discrete, locomotor activity pulses induced during the mid-to-late part of the subjective day lead to phase-advances of free-running rhythms in hamsters. Triazolam, a short-acting benzodiazepine, and confinement to a novel-running wheel also produce this effect in constant conditions (Turek and Losee-Olson, 1986; Turek and Losee-Olson, 1987; Reebs et al., 1989; Reebs and Mrosovsky, 1989a). The PRCs of both triazolam (Turek and Losee-Olson, 1986) and novel wheel-induced locomotor activity pulses (Reebs and Mrosovsky, 1989a) similarly define a classical non-photic profile, with maximal phase-advances of 2-3 h, when activity is induced during the mid-to-late subjective day and somewhat smaller phase delays during the late subjective night (Reebs and Mrosovsky, 1989a, b; Wickland and Turek, 1991). The magnitude of phase-shifts resulting from induced locomotor activity are dose-dependent (i.e., correlated with the amount of locomotor activity performed) (Wickland and Turek, 1991; Janik and Mrosovsky, 1993; Weisgerber et al., 1997; Bobrzynska and Mrosovsky, 1998). Running during 3 consecutive hours is necessary to induce maximal

responses in hamsters (Reebs and Mrosovsky, 1989b; Wickland and Turek, 1991). Therefore, the circadian system of nocturnal rodents has a relatively high threshold for exercise to alter circadian function, at least compared to light, which produces measureable phase-shifts after as little as a few minutes of exposure to low-intensity light (Takahashi et al., 1984; Sharma and Chandrashekaran, 1997). This relatively low sensitivity to locomotor activity may represent a buffering system to prevent inappropriate phase-shifting to weak arousal or small amounts of activity which may be normal outside the main active period.

4.2 Arousal independent of locomotor activity

In studies that implicate high-intensity locomotor activity (forced or voluntary exercise), sleep loss or nonspecific arousal may well participate in the phase-shifting process, independently of locomotion *per se*. Animals that run little after an arousing stimulus may fail to shift because they do not stay awake, whereas the animal that shifts after intense running may do so because it remains awake. This possibility of potential contribution of nonspecific arousal to non-photic stimuli was already mentioned in early experiments on non-photic resetting (Mrosovsky, 1988; Rusak et al., 1989). Furthermore, brief arousing episodes induced by an intraperitoneal (i.p.) injection of saline also produce phase-shifts without substantial locomotor activity, though the magnitude of shifts remains much smaller (Hastings et al., 1998). Antle and Mistlberger (2000) showed in Syrian hamsters that the phase-shifting effects of intense locomotor activity can be fully mimicked by keeping animals awake by gentle handling, with minimal activity (Antle and Mistlberger, 2000). Because sleep deprivation leads to accumulation of extracellular adenosine in the central nervous system, experiments aimed at mimicking sleep deprivation used intracranial or i.p. injection of adenosine A1 agonist. When administered during the mid-sleep period, these adenosinergic compounds produce dose-dependent shifts similar to those induced by sleep deprivation. Accordingly, the adenosine antagonist caffeine attenuates the shifts induced by sleep deprivation (Antle et al., 2001). In addition to these findings, other wake-promoting drugs, such as methamphetamine and modafinil, also attenuate light-induced behavioral phase-shifts in nocturnal rodents (Moriya et al., 1996; Vivanco et al., 2013).

4.3 Interaction between behavioral arousal and photic entraining stimuli

In laboratory conditions, the environmental variables are tightly controlled in order to determine the responses to specific stimuli. By contrast, in nature it will be more common that different *Zeitgebers* act in combination. The responses of the mammalian circadian system to

photic and non-photic stimuli are both mediated by effects on the central pacemaker. Therefore, it is important to consider the convergent, yet distinct, responses of animals to non-photic and photic signals. In this subsection, some findings on the interaction of arousal and photic stimuli on the circadian clock will be presented.

As discussed above, acute arousal due to enhanced physical exercise or sleep deprivation in the middle of rest period induces large phase-advances in nocturnal rodents housed in constant darkness. The same stimulus at this circadian phase has little (Janik et al., 1994) or no detectable effect if the animal is stably entrained to an LD cycle and not transferred to constant darkness immediately after the stimulus. However, in young hamsters naïve to wheel running that are placed in a wheel for the first time in the light portion of LD cycle, an extended bout of running may occur. This procedure has been found, in some cases, to markedly phase-advance the circadian cycle, which then takes several days to re-entrain to LD. Interestingly, if the wheel transfer is performed at the usual time of lights off, no shift is observed (Gannon and Rea, 1995).

The question is why an arousal process stimulated during the middle of the light period normally induces little or no shift of nocturnal activity in animals housed in LD cycle? Do arousal and light stimuli work antagonistically on the circadian phase? Accordingly, phase-advance shifts induced by light pulse are attenuated if they are concurrent or followed by arousing stimuli (Ralph and Mrosovsky, 1992; Mistlberger and Antle, 1998). Furthermore, sleep deprivation or injection of an adenosine A1 agonist in the middle or late light period also inhibits the light-induced phase-shifts of locomotor activity rhythms in mice and hamsters (Watanabe et al., 1996; Mistlberger et al., 1997; Challet et al., 2001; Elliott et al., 2001; Sigworth and Rea, 2003). These inhibitory interactions have been conceptualized as the result of opposite actions of photic and non-photic stimuli on the expression of clock genes in the SCN pacemaker, light activating and behavioral arousal suppressing expression of the clock genes *Per1* and *Per2*, thus reversing the cellular changes that would normally shift the circadian timing loop (Maywood et al., 1999; Maywood and Mrosovsky, 2001).

On the other hand, in some conditions arousing and photic stimuli act synergistically. For instance, Syrian hamsters subjected to an 8-h phase-advance of the LD cycle take about 8 to 10 days to fully re-entrain. However, when they are allowed to run in a novel wheel for the first 3 h of the first advanced dark period, re-entrainment is accomplished within only two cycles (Mrosovsky and Salmon, 1987) (**Figure 12**). Similar results have been also reported in mice (Yamanaka et al., 2008). The mechanism of this effect remains to be determined.

In addition to its acute effect, long-term scheduled arousal also modulates the photic entrainment process. A remarkable example has been obtained by transferring Syrian hamsters from their home cage into a novel wheel for 3 h each day in the middle of the light period, while maintaining the LD cycle (Sinclair and Mistlberger, 1997). This repetition over one week or more gradually induces a characteristic delay in the onset of nocturnal running that may exceed 6 h. This procedure seems to split the circadian activity cycle into two components in some experiments which were investigated by discontinuing the arousal schedule in darkness for few days (Mrosovsky and Janik, 1993; Gorman and Lee, 2001). The neural and molecular events associated with such splits are not clearly understood yet.
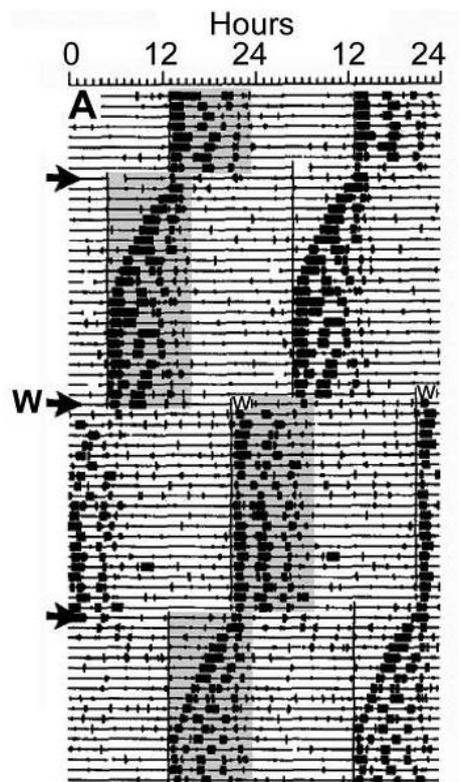
Figure 12. Synergy: 3h activity bouts accelerate re-entrainment to an 8h advance light/dark (LD) cycle

A Syrian hamster entrained to LD takes ~10 days to re-entrain following an 8h advance of the LD cycle (top and bottom arrow), but re-entrains within 2 days if stimulated to run during the first 3 h of the first shifted dark period by transfer to a novel wheel (middle arrow, labelled "W").

Adapted from Mistlberger and Nadeau (1992)

4.4 Pathways and neurotransmitters

The SCN afferents involved in non-photic modulation of circadian rhythms are divided into two major pathways: **1.** The GHT originating in the IGL, which contains NPY and GABA amongst others (Harrington et al., 1985; Moore and Card, 1994) and **2.** An ascending serotonergic pathway originating in the median raphe nucleus (Meyer-Bernstein and Morin, 1996). Emerging evidence also relates some other neurotransmitters and neuropeptides to the

non-photic regulatory mechanism of circadian phase, such as acetylcholine, orexin and neurotensin.

4.4.1 The GHT

The GHT is a thin elongated fiber tract that is situated between the dorsal and ventral lateral geniculate nuclei, arsies from IGL (Hickey and Spear, 1976; Pickard, 1985; Morin et al., 1992), and projects to the ventral SCN (Morin, 2013). Both wheel-running and sleep deprivation by gentle handling markedly increase c-Fos expression in IGL neurons (Janik et al., 1995; Antle and Mistlberger, 2000). Electrical stimulation of the IGL produces phase-shifts, as behavioral arousal does (Rusak et al., 1989). At the same time, it has been reported that ablation of this region blocks the phase-shifts to various means of arousing hamsters and mice (Johnson et al., 1988; Janik and Mrosovsky, 1994; Wickland and Turek, 1994; Koletar et al., 2011). These findings suggest that activation of the IGL is necessary for circadian clock resetting mediated by arousal stimuli. In addition to NPY and GABA the IGL neurons that project to the SCN may also contain enkephalin or neurotensin as neurotransmitter (Morin and Blanchard, 2001).

Neuropeptide Y: In hamsters, almost 50% of IGL neurons that project to SCN are immunoreactive for NPY (Morin and Blanchard, 2001). Wheel confinement in the middle of the rest period markedly increases c-Fos expression in the IGL NPY neurons and increases the release of this peptide in the SCN (Janik et al., 1995; Glass et al., 2010). Remarkably nocturnal light pulses also induce c-Fos in the IGL, but not in NPY neurons (Janik et al., 1995). Intra-SCN administration of NPY results in a PRC similar to that produced by non-photic behavioral manipulations in hamster and mice (Biello et al., 1994; Huhman and Albers, 1994; Maywood et al., 2002; Soscia and Harrington, 2005). Blocking the effect of NPY by injection of NPY antiserum severely attenuates wheel-running-induced phase-advances without reducing wheel revolutions during the novelty pulse (Biello et al., 1994). *In vitro* treatment with NPY during subjective day dose dependently phase-advances the peak firing rate of the SCN neurons (Medanic and Gillette, 1993; Shibata and Moore, 1993; Biello et al., 1997; Harrington and Schak, 2000). Furthermore, both *in vitro* and *in vivo* application of NPY suppresses *Per1* and *Per2* expression (Fukuhara et al., 2001; Maywood et al., 2002). NPY may also be involved in non-photic inhibition of photic response, because NPY infusion to the SCN completely blocks light-induced phase advances (Weber and Rea, 1997).

GABA: Most of the neurons in the IGL (including those projecting via the GHT) and SCN are GABAergic (Moore and Speh, 1993; Moore and Card, 1994; Morin and Blanchard, 2001). $GABA_A$ receptor agonists have been shown to perturb the circadian phase in hamsters and mice. Midday treatment with the $GABA_A$ agonist muscimol induces phase advances of the behavioral rhythm (Ebihara et al., 1988; Smith et al., 1989). Intra-SCN administration of muscimol *in vivo* (Mintz et al., 2002; Ehlen and Paul, 2009) or application of muscimol *in vitro* induces non-photic like phase-shifts in behavioral and SCN electrical activity rhythms (Tominaga et al., 1994; McElroy et al., 2009). SCN $GABA_a$ receptor activation downstream of IGL input is also necessary for NPY-mediated clock resetting since phase-shifts to NPY are blocked by administration of the $GABA_A$ receptor antagonist bicuculline both *in vitro* and *in vivo* (Janik et al., 1994; Huhman et al., 1997; Gribkoff et al., 1998).

Enkephalin: Enkephalin-containing neurons in the IGL have been reported in several species and these neurons also contribute to the GHT (Card and Moore, 1989; Smale et al., 1991; Moore and Speh, 1993; Morin and Blanchard, 1995; Morin and Blanchard, 2001). *In vivo*, systemic administration of fentanyl (a relatively selective μ receptor agonist) has been reported to induce large phase-advances in hamsters when administered during the mid-to late-light period. Like fentanyl treatment a similar phase-advance shifts has been reported after morphine treatment (Chen et al., 1993; Marchant and Mistlberger, 1995; Meijer et al., 2000; Vansteensel et al., 2003; Vansteensel et al., 2005). Enkephalinergic cells have been also found in dorsomedial SCN (Abrahamson and Moore, 2001; Morin and Allen, 2006), suggesting that also release of these neuropeptides from neurons within the SCN may contribute to the control of circadian phase.

Neurotensin: Neurotensin-positive cells have been detected in the hamster IGL and these cells project to the SCN (Morin and Blanchard, 2001). Neurotensin-expressing cells also have been described in the mouse ventral SCN (Abrahamson and Moore, 2001), as well as in the human SCN (Goncharuk et al., 2001). *In vitro* midday (CT6) application of this neuropeptide to the rat SCN induces large phase-advances in neuronal firing (Meyer-Spasche et al., 2002).

4.4.2 Serotonin and raphe nuclei

Serotonin (5-HT) has long been reported to modulate circadian responses. 5-HT transmission has been proposed as a potential mediator of behavioral phase-shifts and modulation of photic synchronization by arousal (Moriya et al., 1996; Sumova et al., 1996; Watanabe et al., 1996; Morin, 1999; Grossman et al., 2000; Challet et al., 2001). The release of 5-HT associated with

arousal and arousal-induced behavioral shifts has an inhibitory effect on photic resetting or entrainment in nocturnal rodents (Moriya et al., 1996; Sumova et al., 1996; Mistlberger and Antle, 1998; Grossman et al., 2000). The phase-shifts induced by arousal are attenuated by pre-treatment with an serotonin antagonist in the rat (Sumova et al., 1996). In hamsters, however, phase-shifts induced by stimulated running are not blocked by 5-HT receptor antagonists or serotonin depletion in SCN (Bobrzynska et al., 1996; Antle et al., 1998). On the other hand, stimulated locomotor activity or sleep deprivation does lead to release of serotonin in the SCN of nocturnal rodents (Dudley et al., 1998; Grossman et al., 2000). The serotonergic projection from the median raphe innervates the SCN, while the IGL receives an serotonergic projection from the dorsal raphe (Hughes and Piggins, 2012). Waking is associated with increased firing rate of serotonergic neurons in the raphe nuclei and increased 5-HT release in the SCN and IGL (Dudley et al., 1998; Jacobs and Fornal, 1999; Grossman et al., 2004). Electrical stimulation of the raphe nuclei leads to behavioral phase-shifts with increased levels of serotonin and suppressed c-Fos expression in the SCN of nocturnal species (Dudley et al., 1999; Meyer-Bernstein and Morin, 1999). The SCN serotonin release associated with arousal-induced phase-shifts is blocked by intra-dorsal raphe injections of $5\text{-}HT_{1,2,7}$ and $5\text{-}HT_7$ antagonist, indicating the role of dorsal raphe output in the phase-resetting response to arousal (Glass et al., 2003) It has been reported that arousal attenuates photic resetting of the SCN clock via associated serotonergic changes (Moriya et al., 1996; Ono et al., 1996; Challet et al., 2001). Furthermore, administration of 5-HT and $5\text{-}HT_{1A\,/7}$ agonists to SCN slices *in vitro,* as well as in whole animals *in vivo* in general produce typical non-photic-like PRCs, with large phase-advances during the day and small, if any, phase-delays in late night (Prosser et al., 1990; Medanic and Gillette, 1992; Shibata et al., 1992; Tominaga et al., 1992; Edgar et al., 1993b; Bobrzynska et al., 1996; Challet et al., 1998; Ehlen et al., 2001; Horikawa and Shibata, 2004).

4.4.3 Orexin and the lateral hypothalamic area

The lateral hypothalamic area (LH) contains orexin-expressing neurons that are critical for the appropriate control of arousal (Sakurai, 2007), and innervate a number of structures implicated in arousal-mediated circadian resetting, such as IGL, median raphe and the peri-SCN region (Hughes and Piggins, 2012) (**Figure 8**). Orexin neurons receive indirect afferent projections from the SCN (Abrahamson and Moore, 2001; Deurveilher and Semba, 2005), suggesting reciprocal modulation between circadian and arousal-promoting circuits. It has been reported that novel wheel-running, sleep deprivation by gentle handling and dark pulses

elevate c-Fos expression in orexin-expressing cells (Marston et al., 2008; Webb et al., 2008). It has been that orexin acutely alters both SCN and IGL neuronal activity *in vitro* (Farkas et al., 2002; Brown et al., 2008; Klisch et al., 2009; Pekala et al., 2011) and activation of orexin neurons in the LH precedes the onset of the main activity bout (Marston et al., 2008). So far, it is not fully clear whether activation of LH neurons is a consequence of non-photic-like phase-shifts or part of the upstream mechanisms controlling them.

4.5 Molecular mechanism and signaling pathways

Arousing stimuli modify the immediate early gene expression in the circadian system. In particular, these non-photic manipulations decrease rhythmic c-Fos expression in the SCN (**Figure 13**), while they induce its expression in the IGL of nocturnal rodents (Janik and Mrosovsky, 1992; Mistlberger et al., 1998; Antle and Mistlberger, 2000; Coogan and Piggins, 2005). In response to non-photic stimuli, c-Fos expression is found specifically in NPYergic cells of the IGL (Janik et al., 1995), whereas a light pulse appears to activate non-NPYergic cells in the IGL. Thus, although the IGL is involved in both non-photic and photic entrainment, it does so through separate cell populations.

As already mentioned, the level of *Per1* and *Per2* mRNA are rapidly down-regulated following daytime wheel confinement (Maywood et al., 1999; Maywood and Mrosovsky, 2001). Similarly, *Per1* levels in the SCN decrease after sleep deprivation (Webb et al., 2014) (**Figure 13**). Other non-photic stimuli such as systemic injection of $5-HT_{1A/7}$ agonist, intra-SCN NPY and dark pulses, also decrease *Per1* and *Per2* expression in the SCN (Horikawa et al., 2000; Fukuhara et al., 2001; Mendoza et al., 2004).

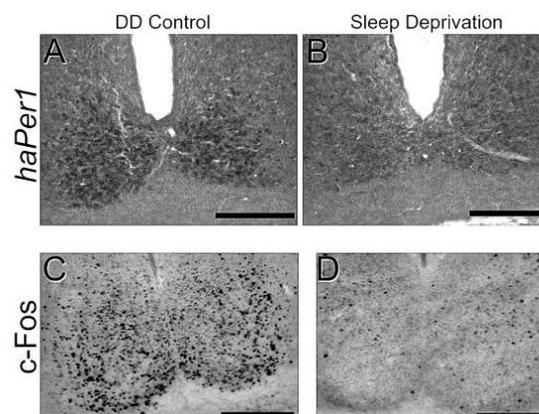**Figure 13. Sleep deprivation inhibits Per1 and c-Fos expression in the SCN**

Hamster Per1 mRNA and c-Fos protein are expressed at high levels in the suprachiasmatic nucleus (SCN) during midday (A, C). Behavioral arousal by 3-hr sleep deprivation rapidly suppresses both Per1 (B) and c-Fos (D) in the SCN. Scale bars = 200µm; DD = Complete dark. *From Webb et al. (2014)*

Serotonergic resetting of the clock is mediated by the 5-HT$_7$ receptor (Lovenberg et al., 1993), and activation of the 5-HT$_7$ receptor leads to an increase in cyclic adenosine monophosphate (cAMP) production. *In vitro* the circadian rhythm in electrical firing rate of the SCN can be shifted in a non-photic manner by activation of the cAMP cascade (Prosser and Gillette, 1989). Protein kinase A (PKA) is activated by cAMP. Phase-shifts to 5-HT$_{1A}$ agonists of *in vitro* SCN preparations can be inhibited by pretreatment of the slice with a PKA inhibitor (Prosser et al., 1994). The non-photic-like phase-shift induced by NPY is mediated by the NPY Y$_2$ receptor (Golombek et al., 1996). Activation of this receptor leads to activation of phospholipase C and production of inositol triphosphate and diacyl-glycerol, which then leads to release of intracellular Ca^{2+} and activation of protein kinase C (PKC). Pretreatment of SCN slices with PKC inhibitors blocks the NPY-induced phase-shifts of the circadian rhythm of electrical firing rate (Biello et al., 1997).

The MAPK family has also been implicated in non-photic and dark pulse phase-shifting. Dark pulses during subjective day and sleep deprivation suppress ERK phosphorylation in the SCN (Coogan and Piggins, 2005). ERK activation may regulate transcription factors, which could lead to changes in clock gene expression. The activated form of ERK is decreased by sleep deprivation in most of the SCN. On the other hand sleep deprivation activates ERK in a small dorsolateral cluster of cells in the caudal SCN (Antle et al., 2008).

4.6 SCN electrical activity suppression and clock resetting

Electrical activity of the SCN shows a circadian rhythm which is correlated with the rest/activity cycle (Inouye and Kawamura, 1979). *In vivo* electrophysiological recordings in both nocturnal and diurnal rodents show that the neuronal firing rate in the SCN increases during subjective day and decreases during the subjective night (Inouye and Kawamura, 1979; Meijer et al., 1997; Meijer et al., 1998; Deboer et al., 2003). In many cases, behavioral arousal or adenosine receptor agonists that phase-shift the circadian clock in a non-photic manner are associated with an inhibition of SCN neuronal activity, at least in nocturnal animals (discussed in 4.4). In freely-moving nocturnal rats and hamsters, increased locomotor activity bouts are associated with a transient reduction of SCN firing rate which recovers when the activity bout ceases (Meijer et al., 1997; Yamazaki et al., 1998; Schaap and Meijer, 2001). This suppression of SCN firing rate is not induced when animals perform less vigorous activities, such as grooming and feeding. The magnitude of the suppression is correlated with the intensity of wheel running (Yamazaki et al., 1998; Schaap and Meijer, 2001). Moreover, NPY, GABA and serotonin predominantly suppress SCN firing rate (Webb et al., 2014). It

would be interesting to study whether behavioral arousal that phase-shifts the circadian clock may also suppress SCN firing rate, independently of locomotor activity.

Recent work has suggested that the ability of these stimuli to suppress neural activity may be crucial for resetting the molecular clock mechanism. Indeed, NPY-induced phase-advances of SCN *Per2* expression *in vitro* are blocked by K^+ mediated depolarization even 2 h after NPY washout (Besing et al., 2012). Thus, long-term inhibition of SCN neural firing may be necessary to induce non-photic circadian resetting.

4.7 Non-photic entrainment: Diurnal species

Unlike light which evokes a similar PRC for both nocturnal and diurnal species, non-photic entrainment shows disparities in terms of the general PRC in nocturnal and diurnal species. As discussed above (section 4.1,4.2), non-photic stimuli and dark pulses induce characteristic phase-advances during the subjective day (resting period) and eventually delays in late subjective night (active period) in nocturnal rodents (Reebs et al., 1989; Reebs and Mrosovsky, 1989a; Antle and Mistlberger, 2000; Rosenwasser and Dwyer, 2002; Challet, 2007). On the other hand, in the diurnal rodents *Arvicanthis ansorgei* and *Octodon degus*, dark pulses during the subjective night induce phase-advances (i.e., corresponding to resting phase in diurnal species) (Lee and Labyak, 1997; Mendoza et al., 2007). Results from these experiments suggest that the circadian sensitivity to dark pulses differs largely between nocturnal and diurnal rodents. At the molecular level, dark pulses down-regulate the expression of *Per1* and *Per2* in the SCN of nocturnal and diurnal rodents (Mendoza et al., 2004). Despite different circadian windows of sensitivity, this apparent similarity of dark-induced molecular effect may be due partly to constant light-induced changes in *Per1* and *Per2* expression, their mRNA levels being still high during early subjective night in the diurnal rodent. The effects of non-photic stimuli such as induced arousal, involuntary locomotor exercise, sleep deprivation, treatment with wake-promoting drugs have not been studied extensively yet in diurnal rodents. Previous studies in the diurnal European ground squirrel and Common marmoset investigated the phase-shifting effect of non-photic activity-inducing locomotor activity (Hut et al., 1999; Glass et al., 2001). Because of small phase-advances observed during the late subjective day, the authors concluded that the responses of the SCN clock to the non-photic stimuli were similar to those in nocturnal rodents. However, this conclusion was based mostly on analysis of the phase-angle of entrainment, not PRC, to single stimuli.

Activation of the $GABA_A$ receptor by muscimol during mid-subjective day induces phase-advances in nocturnal rodents (section 4.4.1), while this GABAergic compound at the same circadian time leads to phase-delays after injection in the SCN of the diurnal unstriped Nile grass rats, *Arvicanthis niloticus* (Smith et al., 1989; Novak and Albers, 2004). Such behabioral shifts induced by $GABA_A$ activation during the mid-subjective day have been associated with a down-regulation of *Per1* and *Per2* in the SCN of nocturnal rodents and only *Per2* in the SCN of diurnal *Arvicanthis* (Ehlen et al., 2006; Novak et al., 2006). Moreover, GABAergic stimulation inhibits light-induced phase-shifts in both nocturnal and diurnal species (Gillespie et al., 1997; Novak and Albers, 2004).

Serotonin is another important neurotransmitter of circadian timing system. SCN serotonin levels follow the daily pattern of activity/rest in nocturnal and diurnal rodents (Poncet et al., 1993; Cuesta et al., 2008). Both *in vitro* and *in vivo* serotonin is capable of phase advancing the SCN clock during the mid-subjective day in nocturnal rodents (Shibata et al., 1992; Bobrzynska et al., 1996). In sharp contrast, in the diurnal rat *Arvicanthis*, serotonergic receptor activation produces phase-advances essentially during the subjective night, but not during the mid-subjective day (Cuesta et al., 2008). In nocturnal rodents, the phase-shifts induced by serotonergic activation are consistently associated with reduced expression of *Per1* and *Per2* in the SCN. However, in the diurnal *Arvicanthis Per* expression remained unchanged, suggesting distinct molecular mechanisms that remain to be identified (Horikawa et al., 2000; Glass et al., 2001; Cuesta et al., 2008). Furthermore, serotonin modulates photic resetting differently between nocturnal and diurnal species. Serotonergic activation reduces light-induced phase-delays and advances in nocturnal hamsters, while this treatment potentiates light-induced phase-shifts in the diurnal *Arvicanthis* (**Figure 14**).
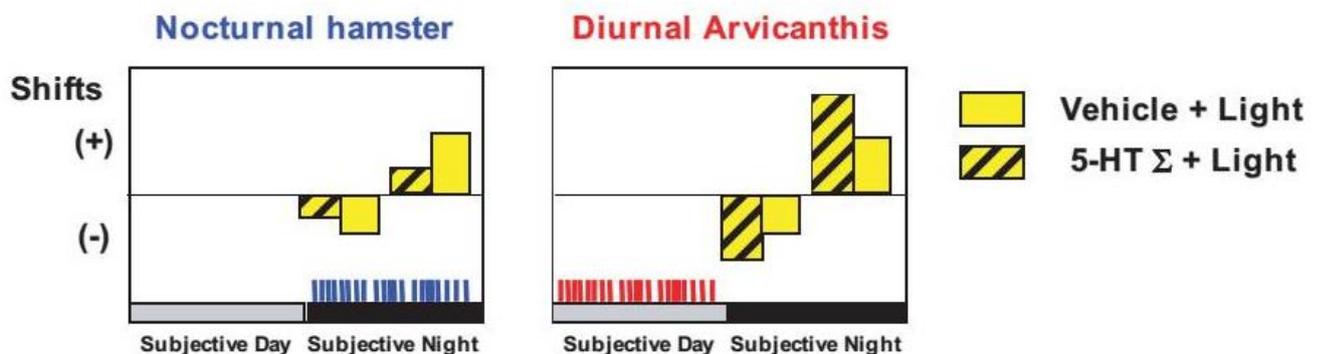

Figure 14. Serotoninergic stimulation modulates photic resetting of clock

Stimulation of serotoninergic receptors reduces light-induced phase-delays and advances in nocturnal hamsters (left side), whereas it potentiates light-induced phase-delays and advances in the diurnal grass rat, *Arvicanthis* (right side).

From Challet, 2007

Effects of non-photic cues on the circadian system have also been investigated in humans. Physical exercise in the early evening induces circadian phase-advances, whereas nocturnal exercise after midnight delays circadian rhythms (Van Reeth et al., 1994; Buxton et al., 1997; Buxton et al., 2003). The combination of exercise and bright light induces significant phase-delays in urinary 6-sulphatoxymelatonin, but does not result in a significant difference in the shifts compared with bright light alone (Youngstedt et al., 2002). On the other hand, another study showed that partial sleep loss reduces bright light-induced phase-advances in humans (Burgess, 2010). Moreover, the effects of caffeine consumption on the human circadian system have also been documented. The study of blind patients suggests that caffeine improves daytime alertness, but fails to entrain their circadian clock (St Hilaire and Lockley, 2015). Nevertheless, caffeine consumption alone and in combination with bright light suppresses melatonin release and attenuates the normal decrease in body temperature in sleep-deprived sighted human subjects (Wright et al., 1997). More recently, it has been shown that evening consumption of caffeine produces significant phase-delays of the plasma melatonin rhythm in dim light, while it does not modify significantly the shifting effect of bright light (Burke et al., 2015).

5. Circadian control of metabolism

5.1. Rhythms of metabolic processes

Energy intake and expenditure fluctuate over a 24 h period in association with sleep/wake, activity/rest and fasting/feeding cycles. Both diurnal and nocturnal animals consume food and water mostly during their wake period, corresponding to either day or night, respectively. Lesion studies have shown that the SCN clock regulates daily rhythmicity of food and water intake (Stephan and Zucker, 1972; Nagai et al., 1978). Like energy intake, energy expenditure in mammals also displays circadian rhythmicity through daily variations in the metabolic rate (Aschoff et al., 1986; Daan et al., 2013). Mammals use ingested and stored calories for the maintenance of basal metabolic rate, resting metabolic rate, core body temperature and physical activities. A significant proportion of energy expenditure goes to the maintenance of the basal metabolic rate which directly affects body temperature. Accordingly, nocturnal rodents show elevated energy expenditure and body temperature in their wake/feeding period in association with behavioural arousal, increased locomotion and food consumption at night (Alberts et al., 2006; Yang et al., 2010) Both core body temperature and energy expenditure display rhythms over the daily 24 h periods that are oppositely phased between diurnal and nocturnal animals (Zulley et al., 1981; Krauchi and Wirz-Justice, 2001; Piccione et al., 2005;

Cuesta et al., 2009). The other determinants of energy expenditure are behavioural arousal and physical exercise, two activities that are also in anti-phase between nocturnal and diurnal species. These parameters also contribute to daily increase in metabolic rate and body temperature during either the diurnal or nocturnal activity phase. Moreover, both behavioural arousal and exercise feedback to the circadian clock in the SCN upon which they act as potent Zeitgebers (Hughes and Piggins, 2012).

Alterations in feeding pattern can desynchronize the metabolic rhythms and eventually disturb energy homeostasis. Feeding regular chow diet during the daytime alters energy balance in nocturnal mice and rats, as compared to animals fed only during the night (Verbaeys et al., 2011; Bray et al., 2013). When mice are forced to eat a high-fat diet during the light phase or at the end of the night, metabolic perturbations occur, inducing increased adiposity and decreased glucose tolerance (Arble et al., 2009; Bray et al., 2010). Furthermore, restricting high-fat feeding only to the active period of animals attenuates body weight gain and improves glucose and lipid metabolism (Hatori et al., 2012; Tsai et al., 2013). Another study suggests that the risk of metabolic disturbances is diminished when caloric intake during the resting period is lower than during the active period (Haraguchi et al., 2014). Concomitant with the metabolic perturbations that occur with the temporal shift in feeding pattern, differential shifts in the phase of circadian gene expression also occur in metabolically active tissues, such as liver, heart, skeletal muscle, and adipose tissue, while the SCN remain unaffected (Damiola et al., 2000; Stokkan et al., 2001). These shifts in peripheral organs result in an asynchrony between the central clock and the peripheral clocks. Restricted feeding outside of an animal's usual activity period not only causes desynchrony between SCN and peripheral organs, but likely also among the peripheral metabolic organs themselves, including liver, skeletal muscle and adipose tissue (Bray et al., 2013).

5.2 Circadian regulation of hormones in nocturnal mammals

5.2.1 Leptin and ghrelin rhythms

Endocrine signals from the periphery exert their effects on the daily energy balance by transmitting signals back to the brain in a circadian-dependent manner. These signals, such as ghrelin and adipokines, provide information to the brain about the presence or absence of peripheral stores or demands in energy. Leptin is an adipokine primarily secreted by the white adipose tissue and transported to, amongst others, the ARC where it inhibits NPY/AgRP neurons and activates POMC/CART neurons, thus resulting in reduced food intake (Kalra et

al., 2003; Sobrino Crespo et al., 2014). The daily rhythm of leptin release is under the control of the SCN clock via its autonomic input to the adipose tissue. Indeed, the leptin rhythm, which peaks in early night in laboratory rats, persists in adrenalectomized animals or during a 6-meals-per-day schedule, but disappears after an SCN lesion (Kalsbeek et al., 2001). The anorectic action of leptin is antagonized by ghrelin which is a hormone primarily secreted by the oxyntic cells of the stomach. Ghrelin evokes feeding primarily through activation of neurons in the ARC, resulting in increased release of NPY and AgRP in the PVN and other hypothalamic target structures (Kalra et al., 2003; Patton and Mistlberger, 2013). Plasma levels of ghrelin oscillate according to the feeding cycle. In nocturnal rodents, plasma levels of ghrelin increase during the resting period (i.e., light phase) in anticipation of food intake (Bodosi et al., 2004; Sanchez et al., 2004), which makes this hormone a putative candidate for regulating the circadian food anticipatory rhythm (Patton and Mistlberger, 2013). Ghrelin also feeds back to the SCN by affecting clock gene expression, producing phase shifts in the fasted state and attenuating light resetting of the SCN (Yannielli et al., 2007; Yi et al., 2008). Therefore, ghrelin may prove to be an endocrine signal involved in the communication between the stomach and the central clock.

5.2.2 Glucocorticoid rhythm

Efferent signals from the SCN help to maintain the daily variations of hormones via neuroendocrine and autonomic outputs which, in turn, can provide temporal cues to targets in peripheral and cerebral tissues. The PVN is an important relay centre for energy homeostasis which receives nervous and diffusible signals from the SCN over the day in a time-dependent manner (Buijs et al., 2003a; Tousson and Meissl, 2004). Neurons containing corticotrophin-releasing hormone (CRH) in the PVN are actually controlled on a daily basis by a balance between inhibitory and stimulatory inputs from the SCN. In turn, CRH triggers secretion of adrenocorticotropic hormone (ACTH) from the pituitary which participates in the daily rhythms of plasma glucocorticoids (Verhagen et al., 2004; Kalsbeek et al., 2008b). In nocturnal rodents, the peak of plasma corticosterone (corticosterone being the main glucocorticoid (GC) in rats) is phase-locked with the activity onset. Circadian regulation of GC secretion from the adrenal gland plays an important role in the regulation of energy metabolism. For example, excess of cortisol (cortisol being the main GC in humans) leads to hyperglycemia, hypertension, sleep disturbance, body weight gain and other metabolic impairments (Carroll and Findling, 2010). GC signalling is critical for maintaining fasting glucose by stimulating hepatic gluconeogenesis (Lin and Accili, 2011). Furthermore,

abnormal activation of GC has diabetogenic consequences, including hyperglycemia (van Raalte et al., 2009). Other studies suggest that GC also directly affect clock gene expression in metabolically active tissues, such as liver and kidney, whereas they can trigger rhythmic gene expression in the liver of SCN-lesioned animals (Balsalobre et al., 2000; Oishi et al., 2005; Reddy et al., 2007). By contrast, in white adipose tissue and liver, adrenalectomy abolishes many daily rhythms in metabolic/adipokine gene expression, but not clock gene expression (Oishi et al., 2005; Su et al., 2014). Moreover, besides clock mechanisms, the GC rhythm can be affected by stress because stressful events occurring during the daily trough of plasma GC (i.e., late night and morning in nocturnal species) will mask the endogenous rhythm of GC. Also, ambient light can acutely modulate secretion of GC. In nocturnal mice and rats, light at different times of day enhances corticosterone release via sympathetic pathways, without inducing ACTH release and independently of the SCN (Buijs et al., 1999; Ishida et al., 2005; Kiessling et al., 2014).

5.2.3 Melatonin rhythm

The daily melatonin rhythm is probably one of the evolutionary oldest hormonal outputs of the circadian timing system (Schippers and Nichols, 2014). In mammals, the SCN tightly regulate the rhythmic synthesis and release of melatonin from the pineal gland. Nocturnal release of melatonin can provide temporal cues to target tissues expressing melatonin receptors. It can also feed back to the SCN clock by activating its receptors expressed therein (Pevet and Challet, 2011). Rhythmic release of GABA from the SCN inhibits sympathetic input from the PVN to the pineal gland during the light period, whereas during the dark period a constant glutamatergic stimulatory input prevails in the absence of the inhibitory GABAergic input and, therefore, leads to secretion of melatonin (Kalsbeek and Fliers, 2013). Furthermore, light exposure at night acutely inhibits synthesis and secretion of pineal melatonin (Redlin, 2001). Consistent experimental results in laboratory rats show that the absence of rhythmic release of melatonin in blood circulation leads to chronodisruption of functions associated with energy metabolism. In particular pinealectomy, suppressing the nocturnal rise in melatonin, disturbs the 24 h rhythm of plasma glucose concentration (la Fleur et al., 2001b) and eliminates the daily rhythm of glucose-induced insulin secretion (Picinato et al., 2002). In addition, impaired glucose tolerance, decreased adipose cell responsiveness to insulin and a reduction in GLUT4 content in muscle and adipose tissue has been reported after pinealectomy (Lima et al., 1998).

5.3 Circadian regulation of glucose homeostasis in nocturnal mammals

Glucose is the fundamental energy substrate, being metabolized by nearly all known living organisms to sustain their life. As energy requirements of the organism fluctuate in a time-of-day-dependent fashion, diurnal oscillations in glucose metabolism are due in part to daily changes in glucose utilization. Here we focus on the circadian regulation of glucose homeostasis and on the disruptive consequences of altered clock functioning on glucose metabolism.

5.3.1 Daily rhythm of glucose metabolism

The glucose concentration in plasma results from coordinated regulation of glucose input (food intake, hepatic glucose production) and its utilization (uptake by skeletal and cardiac muscles, and adipose tissues). A daily rhythm in plasma glucose concentrations has been reported by many authors. But the first evidence for the involvement of the SCN in glucose homeostasis came from the fact that bilateral SCN lesions abolished plasma glucose and insulin rhythms and eliminated the differential day and night response to 2 deoxy-glucose, an inhibitor of glucose utilization (Nagai et al., 1994). Since the rhythms of plasma glucose and insulin concentrations are in phase with the feeding rhythm and insulin secretion is mainly triggered by increased blood glucose in response to food intake, the rhythms of glucose and insulin were long thought to be regulated indirectly through the feeding rhythm.

The first evidence for a direct involvement of the SCN in glucose metabolism came from rats trained to a 6-meals-per-day feeding schedule (i.e., one food access of 10 min every 4 h), as these animals still displayed larger glucose and insulin responses in the evening (Kalsbeek and Strubbe, 1998). Furthermore, the persistence of the daily rhythm of basal plasma glucose concentration in rats fasted or challenged with the 6-meal schedules and its elimination after SCN lesion clearly showed the involvement of the SCN in the regulation of plasma glucose concentration, independently of feeding (La Fleur et al., 1999). On the other hand, plasma insulin and glucagon concentrations increase equally after every meal during the 6-meal feeding schedule, indicating that these hormones do not contribute largely to the genesis of glucose rhythm (La Fleur et al., 1999; Ruiter et al., 2003). The daily rhythm of plasma glucose concentrations in rats shows its acrophase at the end of the light period, anticipating the activity increase at dark onset (La Fleur et al., 1999; Challet et al., 2004; Cailotto et al., 2005). Intravenous glucose tolerance tests at different times of the day demonstrated that glucose disposal shows a daily rhythm which is highest also at the beginning of the dark

period (la Fleur et al., 2001a). Since the glucose concentration in the circulation results from both glucose production and disposal and knowing that both the plasma glucose concentration and glucose disposal is highest at the beginning of activity period, glucose production should be also highest at this point. Thus, in nocturnal animals, glucose production and utilization both increase at the beginning of the activity period and show a clear daily rhythmicity.

Delineation of synaptic connections between SCN and liver through both branches of autonomic nervous system and their involvement in the hyperglycemia induced in animals whose SCN was electrically stimulated, supported the view that the SCN - autonomic nervous system axis is implicated in the regulation of glucose homeostasis (Nagai et al., 1988; Fujii et al., 1989). The effect of hepatic denervations in the rat confirmed the role of SCN in the generation of daily rhythmicity in plasma glucose concentration via its influence on the autonomic innervation to the liver (Kalsbeek et al., 2004; Cailotto et al., 2005). The SCN do not directly target the autonomic motor neurons but instead, they transmit their signals to the autonomic branches through hypothalamic relay structures. The PVN being one of the most important hubs in that respect, because they receive signals from SCN, they integrate and convey them to the peripheral organs via autonomic projections to brainstem and spinal cord (Hosoya et al., 1991; Buijs and Kalsbeek, 2001). The communication between SCN and PVN for controlling plasma glucose concentration has been revealed by administration of $GABA_A$ antagonists and NMDA agonists in the vicinity of the PVN (Kalsbeek et al., 2004).

The results indicated that the hyperglycemia induced by activation of PVN neurons is basically independent of insulin and corticosterone release, while glucagon release might be involved. Moreover, the hyperglycemic effect of the $GABA_A$ antagonist and NMDA was completely prevented by a selective denervation of sympathetic, but not parasympathetic, inputs to the liver (Kalsbeek et al., 2004). Repeating these experiments at different times of the day, as well as in SCN-ablated rats confirmed that the SCN is the main source of these GABAergic and glutamatergic inputs to the PVN. Collectively, these findings demonstrated that activation of PVN neurons results in hyperglycemia through activation of sympathetic inputs to the liver and that pre-autonomic neurons in the PVN are controlled by a balance between these inhibitory and excitatory inputs in order to maintain glucose homeostasis (Kalsbeek et al., 2008a).

Besides SCN control, environmental cues, such as stressful events and ambient light, can also modulate plasma glucose levels. For instance, stress-induced hyperglycemia may blunt the circadian rhythm of plasma glucose. Moreover, plasma glucose in nocturnal rats is increased by light exposure at different times of the day, probably via sympathetic pathways (Challet et

al., 2004). The perifornical (PF) and lateral hypothalamic areas (LH) are other targets of SCN output which harbour metabolic neuropeptides, such as orexin and melanin-concentrating hormone (MCH) (Abrahamson et al., 2001; Deurveilher and Semba, 2005; Valassi et al., 2008). Orexin, which is expressed according to a day/night rhythm, is known to be involved in arousal, food intake and energy metabolism (Zhang et al., 2004; Karasawa et al., 2014; Tabuchi et al., 2014). The activity of orexin neurons in the PF/LH seems to be under the control of rhythmic GABA-mediated inhibition such that orexin release is low during the sleeping period and high during the wake state (Alam et al., 2005).

Activation of orexinergic neurons by administration of a $GABA_A$ antagonist in the PF not only increases arousal, but also enhanced hepatic glucose production, an effect that could largely be prevented by intracerebroventricular pre-treatment with an orexin-1 receptor antagonist (Yi et al., 2009). The activity of orexinergic neurons primarily depends upon sleep and arousal states, but its sympatho-excitatory effects also regulate cardiovascular function, thermoregulation, and energy metabolism, leading to appropriate metabolic adaptation during both sleep and arousal (Grimaldi et al., 2014). To sum up, the SCN clock appears to regulate rhythmicity of glucose production and utilization, as well as insulin release and insulin sensitivity, most likely via projections of the autonomic nervous system to tissues such as liver, muscle and pancreas **(Figure 15)**.
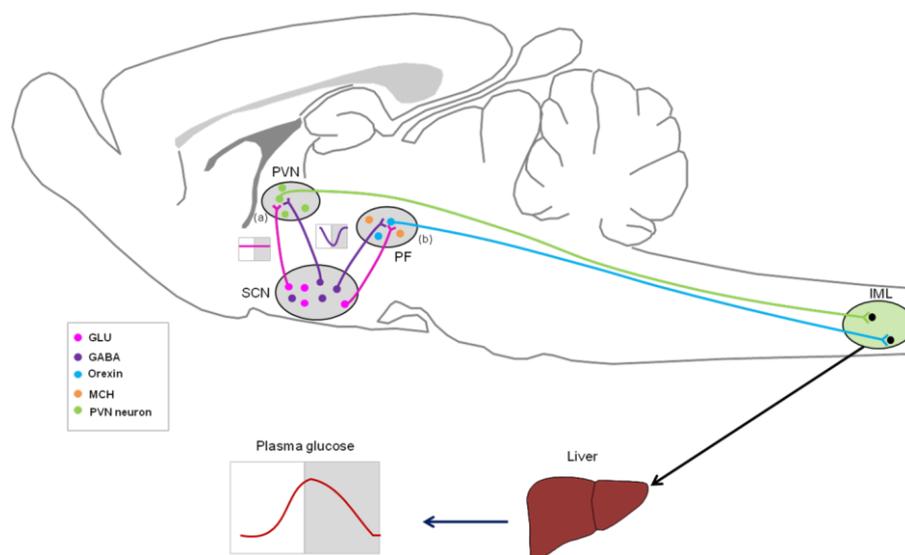

Figure 15 Schematic representation of suprachiasmatic nucleus (SCN) - autonomic nervous system (ANS) axis control over plasma glucose rhythm in the nocturnal rat.

(a) Sympathetic pre-autonomic neurons in the PVN receive a continuous stimulatory glutamatergic input (pink lines) and a rhythmic inhibitory input from GABAergic neurons (purple lines) in the SCN, resulting in the rhythmic activation of sympathetic input (green line) to the liver through the intermediolateral column (IML) of the spinal cord stimulating hepatic glucose production. (b) Orexin-containing neurons in the perifornical area (PF) are also rhythmically activated as a result of continuous stimulatory and rhythmic inhibitory inputs from the SCN. The rhythmic excitatory effect of orexin on the preganglionic neurons in the IML (blue line) controls the daily rhythm of hepatic glucose production via the sympathetic innervation to the liver.

Adapted from Kalsbeek et al.(2010a)

5.3.2 Role of clock components in glucose metabolism

Loss of function of CLOCK or BMAL1, two positive regulators of the molecular clockwork, leads to several metabolic abnormalities. Homozygous *Clock* mutant mice show an attenuated daily rhythm of food intake, as well as hyperphagia and increased adiposity. In addition, they develop metabolic impairments such as hyperglycemia, dislipidemia, hypoinsulinemia, hepatic steatosis and reduced gluconeogenesis (Rudic et al., 2004; Turek et al., 2005). On the other hand, the *Clock* mutation in melatonin-proficient mice does not lead to obesity, but does impair glucose tolerance (Kennaway et al., 2007). Furthermore, the *Clock* gene mutation results in dampened oscillations of hepatic glycogen and glycogen synthase 2 (Gys2), the rate limiting enzyme of glycogenesis, expression (Doi et al., 2010). Mice lacking *Bmal1* in all tissues show impaired glucose tolerance and abnormal energy balance whereas circadian expression of *Glut2* is abolished in hepatocytes of liver-specific *Bmal1* KO mice, in association with relative hypoglycemia during the daily fasting period (i.e., light period) (Lamia et al., 2008). Global *Bmal1* KO mice are also unable to trigger liver gluconeogenesis (Rudic et al., 2004). Mice lacking *Bmal1* specifically in the pancreas or in β cells develop diabetes mellitus due to defective β-cell functioning. These mice show elevated plasma glucose levels, glucose intolerance and loss of glucose-stimulated insulin secretion from β cells. However, insulin content in the pancreatic islets of KO mice is similar to that of wild-type mice, suggesting that insulin secretion, but not its synthesis, is altered in the *Bmal1* mutant mice (Marcheva et al., 2010; Sadacca et al., 2011; Lee et al., 2013).

Glucose homeostasis is also affected by the genetic loss of core clock genes other than *Clock* and *Bmal1*. Disruption of *Per2* expression results in reduced fasting glycemia, altered glycogen accumulation in the liver, enhanced glucose-induced insulin secretion and impaired gluconeogenesis (Schmutz et al., 2010; Zhao et al., 2012; Zani et al., 2013), although Feillet et al. (2006) detected no change in the daily variations of liver glycogen in *Per2* mutant mice. Mice deficient in *Per2* also may have dampened corticosterone rhythms (Yang et al., 2009), but other investigations reported unaltered or increased corticosterone rhythms in *Per2* mutants (Dallmann et al., 2006). Glucose metabolism is also severely affected in *Cry* mutant mice. Genetic loss of both *Cry1* and *Cry 2* (*Cry1$^{-/-}$/Cry2$^{-/-}$*) results in elevated blood glucose levels in response to acute feeding after an overnight fast, and delayed disposal of glucose in a glucose tolerance test (Lamia et al., 2011). A milder impairment in glucose tolerance was detected in mice lacking either *Cry1* (*Cry1$^{-/-}$*) or *Cry2* (*Cry2$^{-/-}$*) (Lamia et al., 2011; Barclay et al., 2013). Moreover, mice overexpressing mutated CRY1 show symptoms of diabetes

mellitus, while overexpression of *Cry1* specifically in the liver of diabetic *db/db* mice improves their insulin sensitivity (Okano et al., 2009; Zhang et al., 2010). In hepatocytes, CRY1 has been found to form a complex with the glucocorticoid receptor and subsequently repress the transcription of phosphoenolpyruvate kinase (PEPCK), the enzyme that catalyses the rate-controlling step of gluconeogenesis (Lamia et al., 2011).

REV-ERBα (also named NR1D1) was initially discovered as a nuclear receptor regulating lipid metabolism and adipocyte differentiation (Fontaine et al., 2003). The modulatory role of REV-ERBα in the molecular clock machinery and its interactions with metabolic transcription factors, such as peroxisome proliferator-activated receptors (PPAR), has established its functional position at the interface between metabolic processes and the circadian clocks (Preitner et al., 2002; Teboul et al., 2008). REV-ERBα may regulate glucose homeostasis through its transcriptional control of the expression of gluconeogenic enzymes in cultured hepatocytes, including glucose-6-phosphatase and PEPCK (Yin et al., 2007). Mice lacking *Rev-erbα* display increased adiposity and mild hyperglycemia over the 24 h cycle, but without insulin resistance (Delezie et al., 2012). *In vitro* down-regulation of *Rev-erbα* by RNA interference treatment in pancreatic β cells or insulinoma cells impairs glucose-induced insulin secretion (Vieira et al., 2012). *Rev-erbα* is highly expressed in oxidative skeletal muscles where it controls muscle mitochondrial content and oxidative function (Woldt et al., 2013). A deficiency in *Rev-erbα* expression in the muscle may impact glucose homeostasis, because after a meal most of the circulating glucose is taken up by skeletal muscles. Muscle-specific *Rev-erbα* KO mice could therefore provide greater insights of the regulation of glucose metabolism by the muscle clock. An overview of metabolic disturbance associated with glucose metabolism in the different global clock gene KOs is summarized in **Table 1**.

Table 1: Changes in glucose and lipid metabolism in whole body clock gene knock-outs

Mutation	Impact on glucose metabolism	Impact on lipid metabolism	References
Clock	Reduced gluconeogenesis and increased insulin sensitivity		Rudic et al., 2004
Clock	Hyperglycemia and hypoinsulinemia	Hypertriglyceridemia and increased serum cholesterol	Turek et al., 2005
Clock	Decreased glucose tolerance	Reduced plasma free fatty acids	Kennaway et al., 2007
Clock	Damped oscillation of hepatic glycogen and glycogen synthase 2 (Gys 2)		Doi et al., 2007
Clock	Increased plasma glucose level and reduced glucose tolerance		Marcheva et al., 2010
Clock		Hypertriglyceridemia and high expression of microsomal triglyceride transfer protein (MTP)	Pan et al., 2010
Clock		Reduced plasma free fatty acid increased cholesterol	Shostak et al., 2013
Bmal1	Glucose intolerance		Lamia et al., 2008
Bmal1	Increased insulin sensitivity	Elevated plasma triglycerides	Rudic et al., 2004
Bmal1	Impaired glucose tolerance and hypoinsulinemia		Marcheva et al., 2010
Bmal1		Elevated circulating free fatty acids	Shimba et al., 2011
Per2	Reduced fasting glycemia and altered glycogen storage		Zani et al., 2013
Per2	Hypoglycemia		Schmutz et al., 2010
Per2	Hyperinsulinemia, Increased glucose tolerance and insulin sensitivity		Zhao et al., 2012
Per 2		Reduced plasma triglycerides and free fatty acids	Grimaldi et al., 2010
Per1/2		Reduced total hepatic triglycerides level	Adamovich et al., 2014
Cry1/Cry2	Increased plasma glucose and reduced glucose tolerance		Lamia et al., 2011
Rev-erbα	Mild hyperglycemia	Reduced plasma free fatty acids and altered rhythmic genes expression related to lipid metabolism.	Delezie et al., 2012
Rev-erbα		Decreased expression of cholesterol-7a-hydroxylase (CYP7A1)	Duez et al., 2008; Le Martelot et al., 2009

5.4 Circadian regulation of lipid homeostasis in nocturnal mammals

Like glucose, also lipid metabolism shows clear daily rhythms in order to fulfil time of the day-dependent energy requirements in association with sleep/wake and fasting/feeding cycles. In this subsection, we summarize what is known about the daily regulation of lipid metabolism by circadian clocks.

5.4.1 Daily rhythms of lipid metabolism

Plasma lipid concentrations as well as their biosynthesis show a clear diurnal rhythmicity. Lipids being hydrophobic molecules cannot circulate readily in the aqueous blood. As a consequence, transportation of lipids via the general circulation depends upon their association with hydrophilic molecules, called apolipoproteins. Lipid molecules, such as triglycerides (TG) and cholesterol, are transported with the help of these apolipoproteins (Hussain and Pan, 2009; Challet, 2013). Experiments in rats and mice suggest that the nocturnal rise in plasma TGs and cholesterol is caused by changes in apoB lipoproteins. Moreover, the intestinal and hepatic expression of the microsomal triglyceride transfer protein (MTP) is also rhythmic and in phase with plasma lipids (Pan and Hussain, 2007). Furthermore, circadian oscillations of plasma TGs in fasted animals strengthen the evidence that clock mechanisms control the diurnal variations of plasma lipids (Fukagawa et al., 1994; Escobar et al., 1998). Interestingly, diurnal variations of both MTP and plasma lipids are altered when mice are kept in constant lighting conditions (light or dark) or subjected to restricted feeding conditions, indicating additional regulation by light and feeding cues (Pan and Hussain, 2007).

Absorption of lipids takes place rhythmically in the intestine. By exploiting a so-called *in situ* loop technique and studying isolated enterocytes, Pan and Hussain (2009) reported that the rate of lipid and cholesterol absorption is higher during the active period and lower during the resting period in nocturnal mice. Intestinal epithelial cells exhibit rhythms in clock gene expression that are synchronized by SCN cues and the availability of food. Specifically, clock gene expression in the intestinal epithelial cells may control the rhythmic expression of different proteins involved in lipid absorption, such as MTP, apolipoprotein A IV and nocturnin (Hussain and Pan, 2014). Circadian oscillations of clock genes are not restricted only to the intestine, but can also be found in other regions and cell types of the gastrointestinal tract (Pardini et al., 2005; Hoogerwerf et al., 2007; Sladek et al., 2007).

Regulation of lipid biosynthesis, transport and their breakdown for energy substrate are firmly associated. Diurnal variations of lipid biosynthesis in the liver, intestine and fat tissues have been reported. Expression of genes involved in TG biosynthesis show circadian rhythmicity (Kohsaka et al., 2007; Kudo et al., 2007; Shostak et al., 2013; Adamovich et al., 2014). For example, sterol regulatory element-binding protein (SREBP)-1c, acetyl co-A carboxylase (ACC), acyl-CoA synthetase (ACSL), fatty acid synthase (FAS) and fatty acid binding protein 4 show diurnal variations in the liver and adipose tissue of mice (Kohsaka et al., 2007; Kudo et al., 2007; Shostak et al., 2013). Recently, Adamovich et al. (2014) reported that wild-type mice fed *ad libitum* show circadian expression of liver enzymes that participate in TG biosynthesis, including Glycerol-3-phosphate acyltransferase (GPAT), 1-acylglycerol-3-phosphate acyltransferase (AGPAT), Lipin and diacylglycerol acyltransferase (DGAT) (Adamovich et al., 2014). In addition to TG, cholesterol biosynthesis also exhibits diurnal variations in the liver and intestine. Several *in vitro* and *in vivo* studies suggest that expression as well as the activity of HMG CoA reductase, a rate limiting enzyme of cholesterol biosynthesis, exhibits a diurnal rhythm (Hamprecht et al., 1969; Shapiro and Rodwell, 1969; Edwards et al., 1972; Mayer, 1976; Mortimer et al., 1998).

Like lipid biosynthesis their breakdown and transport also varies in a time-of-day dependent manner. Circadian oscillations of gene expression of enzymes involved in lipolysis and beta-oxidation of fatty acids have also been reported. For instance, enzymes such as adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), carnitine palmitoyltransferase 1, and medium-chain acyl-CoA dehydrogenase, have been shown to display circadian rhythms at the transcription level in different tissues (Bailey et al., 2014). Lipoprotein lipase (LPL) is an enzyme which cleaves circulating TGs and releases free fatty acids (FFA) for their cellular uptake (Gimble and Floyd, 2009). *Lpl* is intensely expressed in the adipose tissue and skeletal muscle where lipids are stored and utilized, respectively. The activity of LPL displays a diurnal variation which is opposite in phase between adipose tissue and skeletal muscle in a given species.

In nocturnal rats for instance, LPL activity increases during the dark period in adipose tissue (i.e., leading to accelerated fat accumulation), while its activity increases during the light period in the skeletal muscles (i.e., to support increased fat oxidation) (Tsutsumi et al., 2002). Circulating levels of FFA show an elevation during the light period (i.e., during fasting) and decline during the dark period (i.e., during feeding) in nocturnal rats and mice (Tsutsumi et al., 2002; Stavinoha et al., 2004; Shostak et al., 2013). Abrogation of the daily plasma FFA

rhythm in rats with a bilaterally ablated SCN indicates the involvement of the central clock in daily changes in plasma FFA (Yamamoto et al., 1987).

There is evidence that the SCN uses the autonomic nervous pathways to control the day/night rhythms in lipid metabolism, in a similar way as it controls glucose homeostasis. Neuroanatomical tracing studies revealed that adipose tissue is deeply innervated by sympathetic as well as parasympathetic wiring (Bamshad et al., 1998; Kreier et al., 2002). The activation of sympathetic fibers is associated with enhanced lipolysis, whereas parasympathetic denervation leads to reduction in insulin-mediated uptake of glucose and FFA in adipose tissue. Furthermore, in the absence of parasympathetic input, the activity of HSL increased by 51% in the denervated adipose tissue. These findings demonstrate that sympathetic input stimulates catabolism, whereas parasympathetic input governs the anabolic function in the white adipose tissues. The presence of both sympathetic and parasympathetic neurons in the hypothalamus, including the SCN (Kalsbeek et al., 2007), provides clear evidence that the SCN can regulate lipid metabolism in the adipose tissue by exploiting both branches of the autonomic nervous system.

5.4.2 Role of clock components in lipid metabolism

Mutations and KO of clock or clock-controlled genes affects lipid metabolism too. Evidence for the involvement of *Clock* in lipid homeostasis has come from the metabolic phenotype of *Clock* mutant mice that show hyperleptinemia, hypertriglyceridemia and elevated serum cholesterol (Turek et al., 2005). Additional experiments suggest that *Clock* regulates lipid metabolism by affecting lipid uptake, absorption, biosynthesis and breakdown. *Clock* mutant ($Clock^{mt/mt}$) mice that express the dominant-negative protein display high expression of MTP. CLOCK negatively regulates MTP expression by up-regulating small heterodimer partner (SHP) which suppress MTP expression by binding to the Hepatocyte Nuclear Factor 4α (HNF4α)/ liver nuclear receptor homolog 1 (LRH-1) at the MTP promoter (Pan et al., 2010). The genes involved in lipid absorption do not show circadian expression in $Clock^{mt/mt}$ mice and remain irresponsive to restricted feeding (Pan and Hussain, 2009; Pan et al., 2010). *Clock* mutant mice also display altered rhythmic expression of genes involved in TG synthesis and lipolysis (Kudo et al., 2007; Tsai et al., 2010; Shostak et al., 2013).

Loss of BMAL1 in mice leads to a disruption of the daily oscillation of plasma TG (Rudic et al., 2004). BMAL1 also regulates adipogenesis, since mice lacking *Bmal1* show decreases in adipogenesis and expression of several crucial adipogenic or lipogenic factors, such as PPARγ, adipocyte fatty acid–binding protein 2 (aP2), CCAAT/enhancer-binding protein

(C/EBP)α, SREBP-1a, and FAS. On the other hand, overexpression of BMAL1 in adipocytes stimulates the lipid synthesis process (Shimba et al., 2005). Embryonic fibroblasts from *Bmal1* KO mice fail to differentiate into adipocytes. A recent study of Zhang et al. (2014) suggests that BMAL1 promotes *de novo* lipogenesis via insulin-mTORC2-AKT (Zhang et al., 2014). Furthermore, adipose tissue isolated from *Clock*^{-/-} and *Bmal1*^{-/-} mice do not show rhythmic oscillations of lipolytic genes, such as *Hsl* and *Atgl*, which suggest a role of CLOCK and BMAL1 in lipolysis (Shostak et al., 2013). Mice deficient in *Bmal1* show impairments in fat storage as well as utilization. *Bmal1* KO mice display increased levels of circulating FFA, which induces the formation of ectopic fat in the liver and skeletal muscle. At the same time, these mutant mice show high values of their respiratory quotient, suggesting also a role for BMAL1 in the utilization of fat as an energy source (Shimba et al., 2011). A summary of changes in lipid metabolism in the different global clock genes KOs is presented in **Table 1**.

Altered lipid metabolism has also been reported in *Per* and *Cry* deficient mice. Plasma TG levels in mice lacking *Per1* and/or *Per 2* are reduced (Grimaldi et al., 2010; Adamovich et al., 2014). The study in *Per2* KO mice revealed that PER2 exerts its inhibitory action on PPARγ, a master regulator of lipid metabolism, by blocking its ability to recruit promoters (Grimaldi et al., 2010). Deficiency in *Cry* leads to increased susceptibility to diet-induced obesity. *Cry1/2*[-/-] mice challenged with high-fat diet become obese more rapidly as compared to wild-type animals and they show an up-regulation of white adipose tissue genes associated with lipid uptake and lipogenesis, such as Fas, Lpl, Acc1, Acsl4, Dgat1 and -2, and leptin (Barclay et al., 2013).

Mice lacking *Rev-erbα*, the main repressor of *Bmal1*, display impaired lipid and bile acid metabolism as well as adipogenesis (Fontaine et al., 2003; Le Martelot et al., 2009). PPARγ is a prime regulator of lipid metabolism and adipocyte differentiation that also modulates the transcription of *Rev-erbα* (Fontaine et al., 2003). The regulatory function of REV-ERBα is controlled by the nuclear receptor co-repressor 1 (NCoR1) that activates a subunit of histone deacetylase 3 (HDAC3) to mediate transcriptional repression of target genes such as *Bmal1*. Genetic disruption of the NCoR1-HDAC3 association leads to a significant increase in serum ketone bodies and FFA levels, as well as a marked alteration in the daily variation of several hepatic genes involved in lipid metabolism (Alenghat et al., 2008). HDAC3 recruitment to the genome shows a circadian variation in the mouse liver with high and low efficiency during the light and dark periods, respectively. REV-ERBα and NCoR1 recruitments being in phase with HDAC3 recruitment, a low concentration of REV-ERBα decreases the association of HDAC3 with liver metabolic genes in the dark phase, which favours lipid biosynthesis and

storage. On the other hand, in the light phase, high levels of REV-ERBα increase the association of HDAC3 with liver metabolic genes, therefore reducing lipid biosynthesis. Deletion of either *Rev-erbα* or HDAC3 in the mouse liver causes hypertriglyceridemia and liver steatosis (Feng et al., 2011).

The deletion of *Rev-erbα* also leads to increased adiposity with chow feeding, in the absence of significant hyperphagia or hypoactivity. *Rev-erbα*$^{-/-}$ mice show increased susceptibility to diet-induced obesity and alterations of rhythmic metabolic gene expression associated with lipid metabolism. When these KO mice are challenged with fasting for 24h, they preferentially utilize lipids over glucose (Delezie et al., 2012). Genetic loss-of-function and gain-of-function experiments provided evidence that REV-ERBα is involved in the circadian modulation of SREBP activity, and hence the daily expression of SREBP target genes involved in cholesterol and lipid metabolism (Le Martelot et al., 2009). In addition, REV-ERBα also participates in the rhythmic expression of cholesterol-7a-hydroxylase (CYP7A1), a rate-limiting enzyme that converts cholesterol to bile acids (Duez et al., 2008; Le Martelot et al., 2009).

Lipid homeostasis is also perturbed by disruption of clock-controlled genes downstream of the core clock machinery. The ablation of *nocturnin* in mice leads to a lean phenotype with low body weight and reduced visceral fat, even when the animals are challenged with high-fat diet (Green et al., 2007). *Nocturnin* mutant mice (*Noc*$^{-/-}$) absorb less TG after an oral gavage with olive oil (Douris et al., 2011). Gene expression analyses suggest disturbed lipid metabolism and uptake in the *Noc*$^{-/-}$ mice. Genes involved in TG synthesis and storage and chylomicron formation show altered expression, and large cytoplasmic lipid droplets accumulate in the apical domains of enterocytes. Likewise, deficiency of the estrogen-related receptor-α (ERR-α) also results in resistance to high-fat-diet induced obesity and metabolic dysregulation with reduced peripheral fat deposit (Dufour et al., 2011). Finally, also deletion of p75^{NTR} alters the rhythmic oscillation of genes participating in lipid metabolism (Baeza-Raja et al., 2013).

5.5 Circadian regulation of metabolic homeostasis in diurnal mammals

Circadian control of metabolic homeostasis has been extensively investigated in laboratory nocturnal rodents, such as rats and mice. In this subsection we are summarizing circadian regulation of hormone rhythms and glucose and lipid metabolism in diurnal rodents as well as in humans.

5.5.1 Hormonal rhythms in diurnal mammals

In both nocturnal and diurnal rodents, plasma concentrations of leptin show a post-prandial elevation and reach their zenith around the middle of the feeding period and decline thereafter (Kalra et al., 2003; Cuesta et al., 2009) **(Figure 16)**. Circadian variations of circulating leptin have been also reported in humans but unlike rodents, the peak of leptin secretion occurs at night (i.e., during the fasting/sleep period), favoring a decreased appetite state. Meal timing also impacts the daily rhythm of leptin secretion in humans, which is in line with the post-prandial elevations observed in animals (Schoeller et al., 1997; Xu et al., 1999). Post-prandial elevations in plasma leptin are a salient signal to the hypothalamus that peripheral energy demand has been met or is going to be met. In humans an elevation of circulating ghrelin levels is found at night during sleep, while this level declines before awakening in the morning (Cummings et al., 2001). Hence, the midnight peak of anorectic leptin secretion could counterbalance the stimulatory effect of nocturnal ghrelin release. Plasma ghrelin rhythms in diurnal rodents have not been reported yet.

In diurnal and nocturnal species, the peak of plasma GC (corticosterone in lab and Grass rats, and cortisol in humans) is phase-locked with the activity onset, which occurs at dawn and dusk in diurnal and nocturnal mammals, respectively **(Figure 16)**. Vasopressin release from the SCN in the PVN region is high during the light period in both diurnal and nocturnal animals. It is noteworthy, however, that PVN neurons in diurnal and nocturnal rodents respond differently to vasopressin administration. Indeed, vasopressin administered in the PVN of the diurnal Grass rat (*Arvicanthis*) has a stimulatory effect on the release of plasma corticosterone, while the same treatment in nocturnal rats lowers plasma corticosterone levels (Kalsbeek et al., 2008b). These findings highlight that the 12-h reversal of GC and other rhythms between diurnal and nocturnal mammals may be due to an opposite responsiveness of PVN and other SCN target areas. In humans, the cortisol rhythm is affected by perturbations of the sleep-wake cycle (e.g. jet lag, shift work and sleep deprivation), as well as by acute and chronic stress reviewed (Tsang et al., 2013). Moreover, bright light in the morning, but not in the afternoon, nor at night, triggers a significant increase in plasma cortisol (Scheer and Buijs, 1999; Leproult et al., 2001).
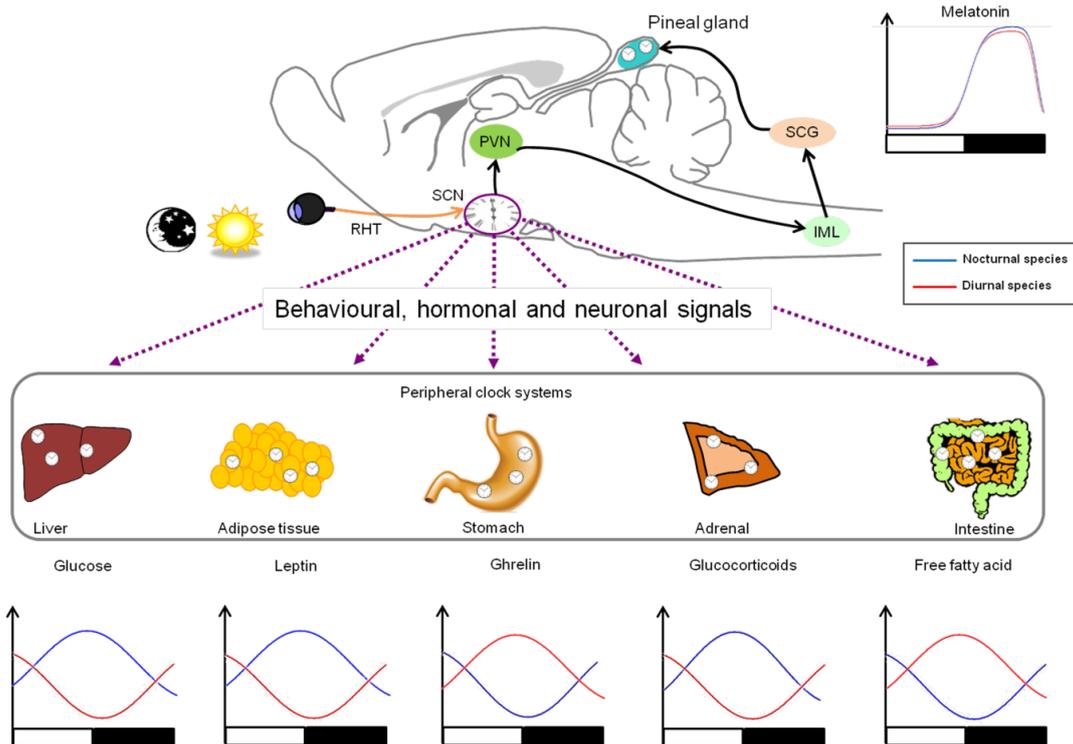
Figure 16 The circadian clock system and its link to hormonal and metabolic rhythms in nocturnal (blue lines) and diurnal (red lines) species.

The mammalian retina receives photic information and conveys it to the suprachiasmatic nucleus (SCN) through the retinohypothalamic tract (RTH). The central clock in the SCN then transmits this environmental cue to extra-SCN clocks in the brain (not shown here) as well as to peripheral tissues via behavioural, hormonal and neuronal signals. The SCN regulates the nocturnal release of melatonin in nocturnal and diurnal species by conveying photic information to the PVN from where autonomic fibers descend to the intermediolateral column (IML) of the spinal cord and then the superior cervical ganglia (SCG) to reach the pineal gland. The SCN communicates through the peripheral clock system in liver, adipose tissue, stomach, adrenal glands and intestine to generate rhythms of plasma glucose, leptin, glucocorticoids, ghrelin and free fatty acids (FFA) that are oppositely phased in nocturnal and diurnal species. Nocturnal rhythms are based on data from rats and mice. Diurnal glucose and leptin rhythm are based on data from the diurnal grass rat, diurnal glucocorticoid, ghrelin and FFA rhythm are based on human data.

One exception to the common scheme of 12-h reversal of hormonal rhythms between diurnal and nocturnal species is pineal melatonin. Unlike the GC rhythm which is oppositely phased between nocturnal and diurnal animals, melatonin is always secreted during the astronomical dark period that corresponds to the active phase in nocturnal, but resting phase in diurnal species (Pevet and Challet, 2011) **(Figure 16)**. Regarding the SCN control of the daily melatonin rhythm in diurnal species, we suggest that it is close to the one described in nocturnal rats, based on the fact that the phase-relationship between the phase of SCN activity (clockwork and firing rate) and melatonin release is similar in nocturnal and diurnal species (i.e., SCN neuronal activity is high when melatonin release is low during daytime, and *vice versa* at night). Because of its sedative effects in humans and its efficiency to treat some sleep disorders, melatonin has been considered as a "sleep" hormone, but this name/function is of

course only correct for diurnal species (i.e., at physiological doses, melatonin does not promote sleep in nocturnal species as they are awake). As mentioned earlier, bright light during nighttime inhibits secretion of pineal melatonin. This observation has been confirmed in all species studied so far, including in humans (Redlin, 2001).

5.5.2 Circadian regulation of glucose metabolism in diurnal mammals

Like in nocturnal mice and rats, plasma glucose concentrations show a day-night rhythm in diurnal rodents as well as in humans, but this rhythm is oppositely phased between nocturnal and diurnal animals **(Figure 16)**. Daily rhythmicity of plasma glucose in the diurnal Sudanian grass rat (*Arvicanthis ansorgei*) shows an acrophase before activity onset (Cuesta et al., 2009). Levels of plasma glucose in human subjects also show a daily rhythm, with a peak before the beginning of wake period, the so-called "dawn phenomenon" (Bolli et al., 1984; Arslanian et al., 1990). In diurnal horse and sheep, however, plasma glucose concentrations peak around mid-night and at dusk, respectively (Piccione et al., 2005). Understanding the mechanisms that underlie these inter-species differences need further investigation. Studies in human subjects indicate that glucose tolerance and insulin sensitivity also vary throughout the day. Both glucose tolerance and insulin sensitivity are more efficient in the morning compared to the evening (Van Cauter et al., 1997).

5.5.3 Circadian regulation of lipid metabolism in diurnal mammals

Daily variations of plasma lipids are robust and in anti-phase, between nocturnal rodents and diurnal humans **(Figure 16)**. Human subjects show higher levels of plasma FFA at night due to enhanced lipolytic activity and this variation persists during fasting (Carroll and Nestel, 1973; Schlierf and Dorow, 1973; Gibson et al., 1975). When minimizing the indirect effects of calorie intake and sleep, by maintaining human subjects in constant dim light, feeding them with hourly isocaloric meals and keeping them awake for 40 hours, Dallmann et al. (2012) reported maintenance of rhythmic plasma FFA levels. In other diurnal animals, like goat and horse, FFA levels are decreased at night and rise in early morning, whereas this rhythm eventually disappears in constant darkness (Orme et al., 1994; Alila-Johansson et al., 2004).

6. Circadian desynchronization

6.1 Deleterious effects of circadian desynchronization on metabolic health

Humans being diurnal, our daytime activities include feeding, work and exercise, while sleep normally takes place during the night-time. An appropriate synchrony between the timing of

our internal clocks and the daily activities helps to maintain metabolic homeostasis. However, the structure and functioning of today's society compel us to loosen this circadian synchrony by increasing night activities, such as shift work and night snacking, by delayed or disturbed sleep, or by travelling frequently across different time zones. Increasing evidence from both epidemiological studies and laboratory experiments indicates that forced circadian misalignment disrupts metabolic rhythms and may thus lead to metabolic abnormalities, such as obesity, type 2 diabetes mellitus, and cardiovascular diseases (Sookoian et al., 2007; Scheer et al., 2009; Salgado-Delgado et al., 2013). Leproult et al., (2014) showed the adverse effects of circadian misalignment on glucose metabolism and cardiovascular health in healthy human subjects. Animal studies support this observation as forced feeding and activity during the usual sleep phase impaired metabolic homeostasis and led to increased body weight gain in rodents (Arble et al., 2009; Salgado-Delgado et al., 2013). In humans, the night-eating syndrome is characterized by the voluntary or uncontrolled food consumption during the night, which is closely related with sleep-related eating disorders. Noteworthy, night-eating syndrome is associated with obesity, as well as hormonal and neurochemical disturbances (Zawilska et al., 2010; Gallant et al., 2012). Moreover, medical students who live a nocturnal life and consume a majority of their food at night display an attenuation of their night-time plasma melatonin and leptin peaks and an impaired response of insulin to glucose, changes that are consistent with those in patients with night-eating syndrome (Qin et al., 2003). Furthermore, dysregulation of the circadian clock in adipose tissue and/or misalignment of meal time may lead to disrupted expression patterns of enzymes involved in lipid metabolism and perturb fatty acid homeostasis (Maury et al., 2010).

Besides experimental studies investigating the role of circadian desynchronizations and their impact on energy metabolism, genetic variations in circadian genes in humans are also associated with effects on glucose and lipid homeostasis. *Clock* and *Bmal1* gene polymorphisms in humans suggest that genetic variation in these genes may play a role in the development of obesity and type 2 diabetes (Woon et al., 2007; Scott et al., 2008; Sookoian et al., 2008). Single-nucleotide polymorphisms (SNPs) in CLOCK are also correlated with elevated plasma ghrelin levels, reduced sleep duration and increased energy intake (Garaulet et al., 2010; Garaulet et al., 2011). Furthermore, carriers of CLOCK SNPs display lower plasma glucose concentrations and improved insulin sensitivity when they eat a diet rich in mono-unsaturated fatty acids (Garaulet et al., 2009; Garcia-Rios et al., 2014). Polymorphisms in the *Per2* and *Npas2* genes have also been associated with high fasting plasma glucose levels (Englund et al., 2009). Moreover, SNPs in loci in or near the *CRY2* gene have been

associated with high fasting glucose levels in non-diabetic participants (Dupuis et al., 2010; Liu et al., 2011). *Rev-erbα* gene polymorphisms in obese subjects are correlated with adiposity, but not plasma glucose and plasma insulin levels (Goumidi et al., 2013). Finally, genome-wide association studies indicate that polymorphisms in the melatonin receptors MTNR1A and MTNR1B, which are expressed in the SCN but also in many peripheral tissues, are linked to increased plasma glucose levels and risk of type 2 diabetes (Bouatia-Naji et al., 2009; Lyssenko et al., 2009; Prokopenko et al., 2009; Li et al., 2011). Altered oscillations of mRNAs encoding circadian regulatory proteins within human subcutaneous adipose tissue are correlated with increased risk of obesity (Wu et al., 2009). Specifically, CLOCK gene polymorphisms are associated with metabolic syndrome, whereas REV-ERBα polymorphisms seem to modulate adiposity in both adult and young people (Garaulet and Madrid, 2009; Goumidi et al., 2013). Although there is no complete mechanistic explanation yet for these observations, they clearly pinpoint the adverse effects of a misalignment between circadian rhythmicity and energy homeostasis.

SCOPE OF THESIS

Circadian rhythms in mammals can be synchronized to local environmental time by the phase resetting action of light cues on the main circadian pacemaker located in the hypothalamic suprachiasmatic nucleus (SCN). The SCN controls the daily rhythms of behavior and metabolism in nocturnal and diurnal animals that in most cases are oppositely phased relative to the light-dark cycle. The behavioral and metabolic cues may also feedback to the SCN clock, influence photic entrainment, as well as physiological and metabolic homeostasis. The aim of this thesis is to elucidate the crosstalk between behavioral arousal, the SCN clock and metabolic homeostasis.

In Chapter 2 we investigate the SCN control of glucose metabolism. Although several SCN neuropeptides, such as vasopressin, VIP and GRP, have been implicated in the brain control of glucose homeostasis, none of them have yet is connected to the SCN control of glucose metabolism. Of note, vasopressin is rhythmically released from the SCN and may thus regulate glucose homeostasis and its rhythmicity by conveying time cues to the metabolic organs via nervous or humoral signals. Here we investigated the effect of central GRP on glucose metabolism by using intracerebroventricular infusions and the stable isotope dilution technique in rats.

Behavioral arousal during the usual resting period of animals affects the daily rhythmicity and homeostasis of metabolic processes. In physiological circumstances, glucose tolerance shows a diurnal pattern, i.e. glucose utilization is highest at the beginning of active period and lowest at the end of active period. In Chapter 3 of this thesis we investigated the effect of acute sleep deprivation on glucose metabolism. For this purpose we performed an intravenous glucose tolerance test (IVGTT) in rats sleep-deprived for 4 h in the early and late subjective resting phase, immediately after the period of sleep deprivation. We also measured the responses of the glucoregulatory hormones (i.e., insulin and corticosterone).

As discussed in the Introduction, arousing stimuli in the sleeping period feedback the clock and results in behavioral phase-shifts and slows down photic entrainment in nocturnal rodents. Sleep/wake cycles of nocturnal and diurnal species are oppositely phased with reference to astronomical day and night. Therefore, in Chapter 4 we investigated whether behavioral arousal in an animal's resting period affects the SCN clock and light resetting similarly or differently in nocturnal and diurnal species. In the Sudanian grass rat (*Arvicanthis ansorgei*), a diurnal rodent, we investigated the behavioral phase-shifts induced by sleep deprivation or caffeine treatment during the resting period. We also investigated the interaction between these arousing stimuli and photic resetting of the SCN clock. Finally, we attempted to understand the cellular and neurochemical basis of the observed behavioral phase-shifts by assessing c-Fos expression and phenotyping the c-Fos positive cells in the SCN.
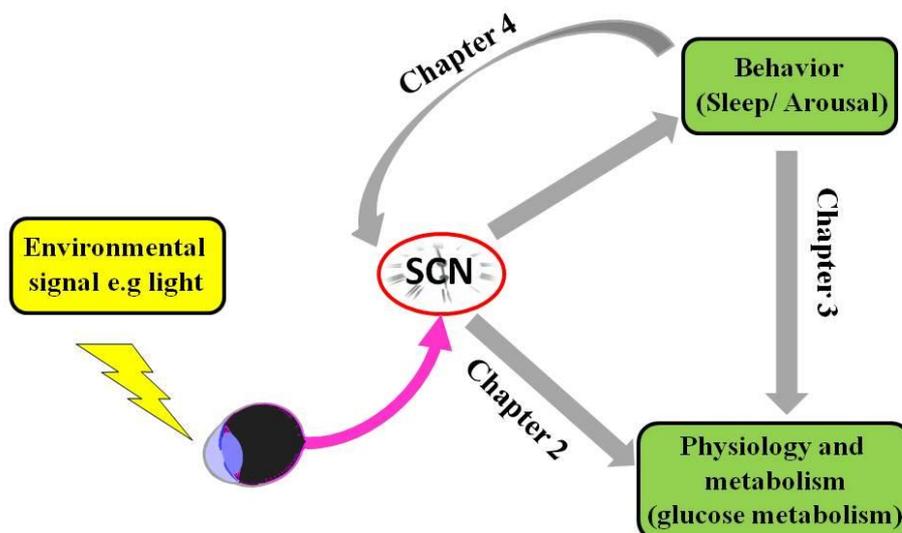

Figure 17. Schematic representation of the circadian clock system including the functional crosstalk between the SCN clock, arousal and metabolism that define the following chapters of this thesis

Finally, the broader implications of the results in these three chapters are addressed with their prospectives in the general discussion section.

Chapter2

Effects of central gastrin-releasing peptide on glucose metabolism

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Abstract

Gastrin–releasing peptide (GRP) mediated signals in the central nervous system (CNS) influence many functions associated with energy metabolism. The purpose of the present study was to investigate the central effect of GRP on glucose metabolism in the male rat. Intracerebroventricular (icv) administration of GRP caused an immediate hyperglycaemia which was sustained till the end of the infusion. The rise in plasma glucose levels was accompanied by an increase in endogenous glucose production (EGP), as well as increases in plasma glucagon and insulin concentrations. Furthermore, no differences in plasma corticosterone levels were noted between control and GRP treated rats. These results demonstrate that central GRP increases plasma glucose levels, probably by stimulating pancreatic glucagon release and concomitantly or subsequently endogenous glucose production.

Key words: Hypothalamus, Suprachiasmatic nuclei, Paraventricular nuclei, Endogenous glucose production, Glucagon

1. Introduction

The plasma glucose concentration results from a coordinated regulation of glucose input (food intake, hepatic glucose production) and its utilization (uptake by skeletal and cardiac muscles, brain and adipose tissues). Plasma glucose concentrations show a daily rhythm that is maintained by the circadian clock in the suprachiasmatic nuclei of the hypothalamus (SCN), in both nocturnal and diurnal mammals (Kumar Jha et al., 2015). The SCN transmit their timing signals via the autonomic nervous system through hypothalamic relay structures, such as the hypothalamic paraventricular nucleus (PVN) and the orexin-containing neurons in the perifornical area (PF). Activation of these nuclei by SCN signals causes hyperglycemia via sympathetic inputs to the liver (Kalsbeek et al., 2004; Yi et al., 2009). Therefore, time-of-day dependent excitatory and inhibitory signals from the SCN to PVN and PF help to maintain the daily rhythm of plasma glucose concentration (Alam et al., 2005; Kalsbeek et al., 2008a; Yi et al., 2009).

In addition to orexin, many other neuropeptides have been implicated in the brain control of glucose homeostasis (Brown, 1981; van Loon and Appel, 1981; Plamondon and Merali, 1993). Also the SCN itself contains a number of neuropeptides that show a daily rhythm in their release pattern, such as arginine vasopressin (AVP) and vasoactive intestinal peptide (VIP), but also gastrin-releasing peptide (GRP) (Chun et al., 1998; Nagai, 2004; Karatsoreos et al., 2006; Aoyagi et al., 2007; Bechtold et al., 2008; Francl et al., 2010; Kalsbeek et al., 2010b). These neurotransmitters might communicate time-cues to metabolic organs via nervous and humoral signals in order to regulate metabolic homeostasis and its daily rhythmicity. Indeed, deletion of the AVP receptor V1a leads to impaired glucose homeostasis in these knock-out mice, whereas mice lacking VIP or the VIP receptor VPAC2 express disrupted behavioural and metabolic rhythms (Aoyagi et al., 2007; Bechtold et al., 2008; Hannibal et al., 2011). GRP is a 27-amino acid neuropeptide and is a mammalian homologue of the amphibian tetradecapeptide bombesin (Bn). It acts by binding to the GPR receptor (GRP-R, a G protein-coupled receptor also known as BB2). The GPR-R preferentially binds GRP (Roesler and Schwartsmann, 2012). GRP-mediated signals influence many functions in the central nervous system, including food intake, glucose metabolism and body weight. In particular, intracerebroventricular (icv) infusions of Bn and Bn-like peptides have been shown to induce anorexia, hypothermia and hyperglycemia in rats (Brown et al., 1977a; Brown et al., 1977b; Plamondon and Merali, 1993; Plamondon et al., 1998; Tsushima and Mori, 2005). GRP-containing neurons originating in the SCN project to the PVN, which is an integrator of

SCN signals and other metabolic cues (Kalsbeek et al., 1993). Additionally, both peripheral and central administration of Bn strongly activates PVN neurons, as shown by increased c-FOS staining (Li and Rowland, 1996). This Bn-induced activation of PVN is lacking in GRP-R KO mice (Ladenheim et al., 2002). In addition, both GRP-R levels and its binding exhibit a daily rhythm in the mouse SCN, with an acrophase at the beginning of the activity period (Karatsoreos et al., 2006).

In the present study, we investigated the possible involvement of GRP in the control of the plasma glucose homeostasis using icv infusions of GRP and the stable isotope dilution technique to determine endogenous glucose production (EGP).

2. Results

Experiments were conducted according to the experimental protocol outlined in **Figure 1**. The icv infusion of GRP resulted in a marked elevation of blood glucose levels (*Infusion* $F_{1,140} = 16.143$, p = 0.001; *Time* $F_{10,140} = 9.677$, p < 0.001; *Infusion*Time* $F_{10,140} = 5.537$; p < 0.001, **Figure 2**). Post-hoc analysis showed that GRP administration increased blood glucose levels at all-time points (t=130 to t=270) from the beginning till the end of the infusion (p < 0.05).
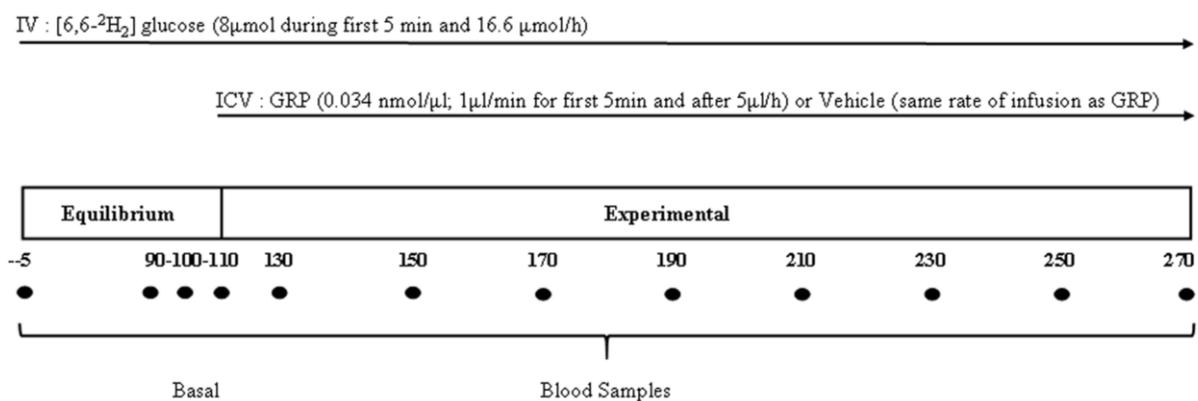

Figure 1 Experimental protocol showing the time line of the infusions (IV and ICV) and the timing of blood sampling. The numbers above the black dots indicate the timing of blood sampling in minutes.
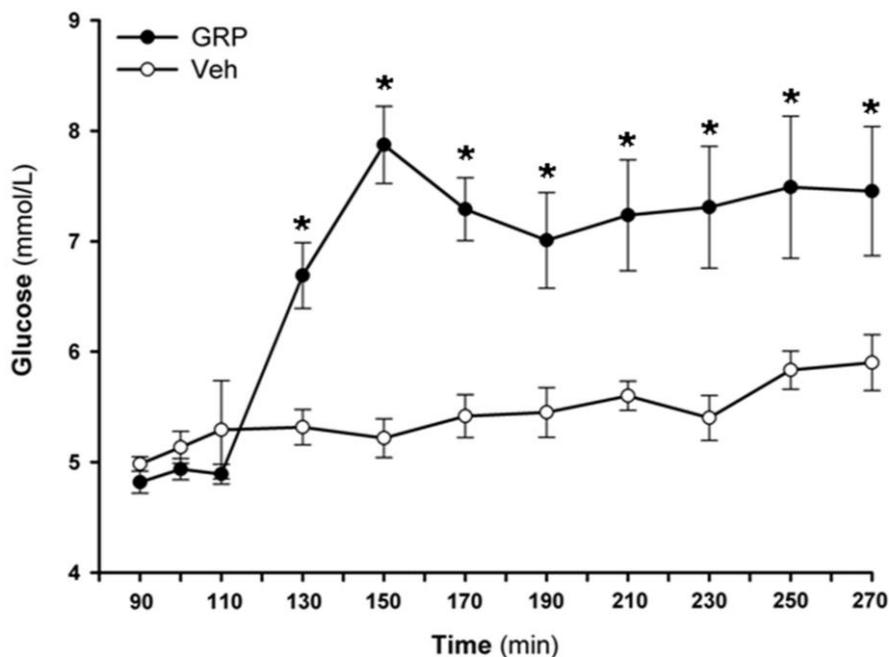
Figure 2 Changes in blood glucose concentration during icv infusion of GRP. The icv infusion of GRP significantly increases blood glucose levels. Veh, vehicle. Data are presented as mean ± SEM. * p < 0.05.

The observed GRP-induced hyperglycemia could be established via different mechanisms. In order to better understand this mechanism, we simultaneously determined endogenous glucose production (EGP), using the stable isotope dilution technique. rmANOVA indicated significant effects of *Time* ($F_{10,139} = 4.531$, p < 0.001) and *Infusion*Time* ($F_{10,139} = 3.816$, p < 0.001) (**Figure 3A**). Post-hoc analysis revealed that in the GRP group EGP was significantly increased as compared to the vehicle group at t = 130 min and t = 150 min (p = 0.006 and p = 0.003, respectively).

As the icv infusion of GRP affected plasma glucose levels and EGP, we also measured the plasma concentrations of the glucoregulatory hormones insulin, glucagon and corticosterone. rmANOVA analysis of plasma insulin levels showed significant effects of *Time* ($F_{4,56} = 10.006$, p < 0.001) and the interaction of *Infusion*Time* ($F_{4,56} = 2.674$, p < 0.04) (**Figure 3B**). Post-hoc analysis showed a significant rise in plasma insulin levels at t = 150 min (p < 0.009). Similar to insulin, rmANOVA analysis of plasma glucagon levels showed significant effects of *Time* ($F_{5,70} = 3.683$, p = 0.005) and *Infusion*Time* ($F_{5,70} = 2.669$; p = 0.029) (**Figure 3C**). Post-hoc analysis showed a significant rise in plasma glucagon levels at t = 130 min (p < 0.022). Plasma corticosterone levels were not different between the GRP and vehicle infused animals (*Time* $F_{10,137} = 1.068$, p = 0.391; *Infusion* $F_{1,137} = 0.208$, p = 0.656; *Infusion*Time* $F_{10,137} = 1.124$, p = 0.349) (**Figure 3D**).
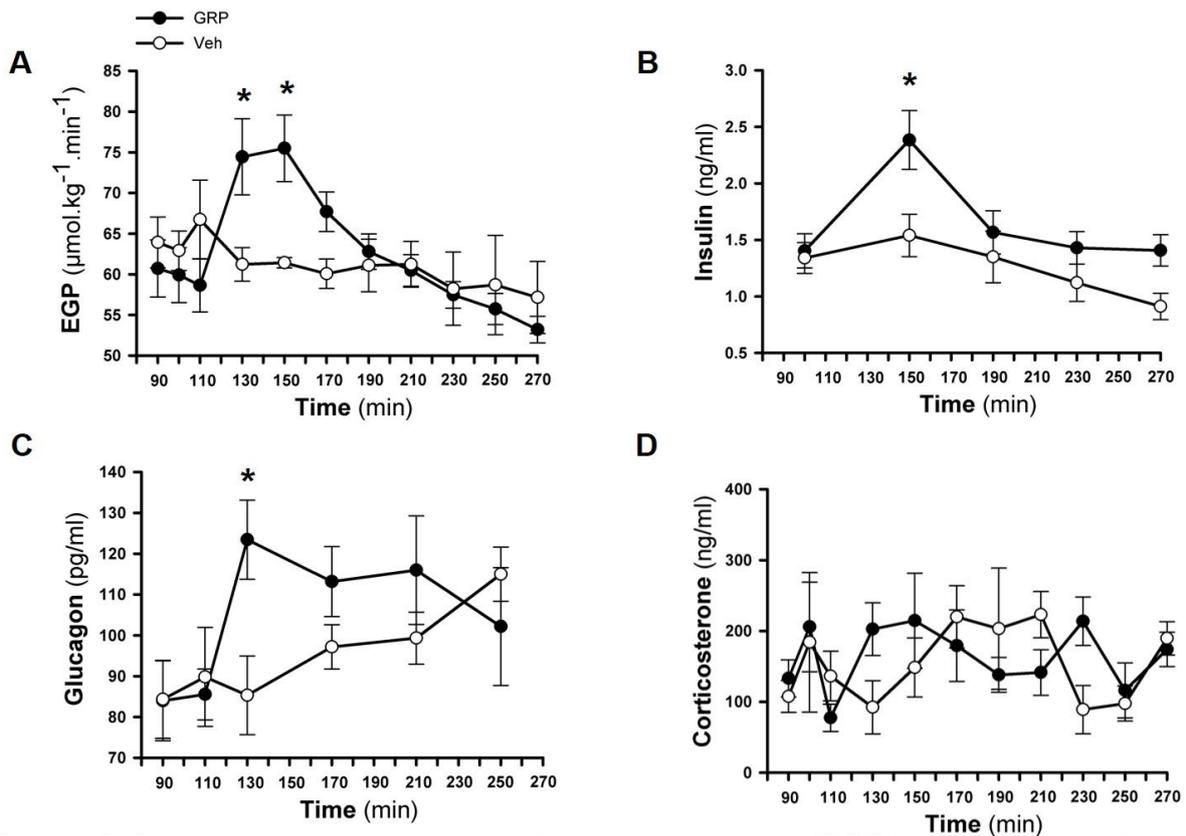
Figure 3 Variations in endogenous glucose production (EGP) and glucoregularory hormones during icv infusion of GRP. The icv administration of GRP significantly increases EGP (A), plasma insulin (B) and glucagon (C). Plasma corticosterone levels remain unchanged (D). Veh, vehicle. Data are presented as mean ± SEM. * p < 0.05

3. Discussion

The present study shows that icv administration of GRP in male rats results in an immediate and persistent hyperglycemia. This result is in line with previous observations showing that central administration of Bn and Bn-like peptides elevate plasma glucose levels in the rat (Brown et al., 1977a; Brown et al., 1977b; Plamondon and Merali, 1993), but by using the stable isotope dilution technique our study was able to demonstrate that the GRP-induced hyperglycemia results mainly from an increase of EGP, i.e., increased gluconeogenesis and/or glycogenolysis.

GRP is widely distributed in the central nervous system (CNS) and peripheral tissues. GRP-R knock-out mice show impaired glucose tolerance and reduced glucagon-like peptide 1 and insulin responses to gastric glucose (Persson et al., 2000).In addition, GRP potentiates the insulin secretion *in vivo* in different species including mice, rats and humans as well as *in vitro* from isolated pancreas of dog (Wood et al., 1983; Pettersson and Ahren, 1987, 1988; Hermansen and Ahren, 1990; Karlsson et al., 1998). These studies suggest that GRP-R signalling in the peripheral tissues affects glucose metabolism. However, GRP-mediated

signals in the CNS can also be involved the regulation of glucose homeostasis. Therefore, to differentiate between the peripheral and central effects of GRP on glucose metabolism, we investigated the consequences of central administration of GRP on plasma glucose and EGP. We observed an increase in plasma glucose levels after icv infusion of GRP. Since both GRP and vehicle infused animals were deprived of food during the experiment itself (i.e., from ~07.30 AM onwards), this increase cannot be explained by an increased food consumption. On the other hand, at the start of the GRP infusion a strong increase in EGP was observed. Therefore, the increased EGP is the most likely explanation for the GRP-induced hyperglycemia. Another way to explain the observed hyperglycemia in the GRP group would be a decrease in glucose uptake. However, global GRP-R knock-out mice show a slight impairment of glucose tolerance after the gastric administration of glucose, accompanied by a reduced insulin response (Persson et al., 2000; Ahren, 2006). Thus GRP would be expected to increase glucose tolerance. In order to investigate the mechanism of icv GRP on glucose uptake in a more quantitative way, hyperinsulinemic-euglycemic clamp experiments in the current paradigm and/or glucose tolerance tests in brain-specific GRP-R knock-out mice should be performed. Moreover, in future experiments pre-treatments with different Bn antagonists prior to GRP administration could provide further information about the specific Bn receptor subtype involved in the GPR effects on glucose metabolism. In addition, peripheral versus central pre-treatment with GRP-R/BB2 antagonist could further strengthen the central action of GRP in the observed hyperglycemia.

In the current experiment, we found no significant difference between plasma corticosterone levels of the vehicle- and GRP-treated animals, ruling out an activation of the hypothalamic-pituitary-adrenal (HPA) axis by GRP. However, results from both *in vitro* and *in vivo* studies suggest that central treatment with GRP influences the HPA axis. GRP acting centrally on GRP-R can increase the secretion of ACTH and corticosterone (Garrido et al., 1998; Garrido et al., 1999). On the other hand, central GRP-R activation in mice by the pro-adrenomedullin N-terminal 20 peptide (PAMP) had no effect on plasma corticosterone levels (Ohinata et al., 2000), which is in line with our data.

While peripheral activation of GRP-R leads to stimulation of insulin secretion in mice, conflicting results have been reported on plasma insulin levels after stimulation of central GRP receptors. Studies in the late seventies by Brown et al. suggest that central administration of Bn exerts a hyperglycemic effect and a relative or absolute decrease in plasma insulin levels, which is abolished after adrenalectomy (Brown et al., 1979; Brown, 1981). However, a bolus icv infusion of Bn in conscious dogs produced an increase in plasma

glucose levels without a significant increase in plasma insulin levels; whereas continuous icv infusion increased plasma insulin levels (Yavropoulou et al., 2010). In our study, plasma insulin levels showed an elevation 40 min after icv GRP and declined to basal levels thereafter. In principle, the GRP infusion could activate the parasympathetic branch of autonomic nervous system and lead to hyperinsulinemia, although parasympathetic activation by central GRP has not been reported before. Moreover, an increased release of insulin would be expected to result in lower levels of plasma glucose, not hyperglycemia. Alternatively and more likely, the currently observed elevation in plasma insulin levels is a direct response to the increased plasma glucose levels.

Most likely the increased plasma glucagon levels considerably contribute to the observed hyperglycemia and increased EGP in the GRP infused group. A previous study by Ohinata et al. (2000) showed that central activation of GRP-R by PAMP results in hyperglycemia, as well as hyperglucagonemia. Consistently, this hyperglycemic effect was inhibited by the co-administration of a GRP-R antagonist. Moreover, peripheral pre-treatment with an α-adrenergic blocker inhibited the PAMP-induced rise in plasma glucose and glucagon, while also pentobarbital pre-treatment (which suppresses activation of the sympathetic nervous system) reduced the hyperglycemic response (Ohinata et al., 2000). Our results are in line with the idea that central GRP stimulates the release of glucagon, which subsequently results in an increased EGP and plasma glucose levels.

The biological effects mediated by the central action of GRP-R involve various aspects of energy metabolism regulation, including effects on food intake, body weight and glucose metabolism. The mechanistic details of the anorexigenic and glucoregulatory actions of GRP are still not clearly understood so far, since GRP-producing neurons can be found in several (hypothalamic) brain areas, including the SCN and PVN. In the PVN, GRP-containing neurons overlap with neurons that contain neuropeptide Y- and melanocortin 4 receptors. Furthermore, altered expression of GRP mRNA in the PVN in response to food deprivation and stimulation of melanocortin receptors suggest that GRP-containing neurons in the PVN may be part of the hypothalamic neuropeptide circuitry that participates in energy homeostasis (Ladenheim et al., 2009). Of interest, Guzman-Ruiz et al (2014) reported that the SCN regulates the activity of α-MSH neurons in the arcuate nucleus (ARC) in a rhythmic fashion via direct GRP-containing projections (Guzman-Ruiz et al., 2014). Also other publications have shown that GRP signalling modulates ARC activity. For instance, Bn-like peptides increase the firing rate of both neuropeptide Y and pro-opiomelanocortin neurons in the arcuate nucleus (Lin and Pan, 1994; van den Pol et al., 2009).

The SCN pacemaker uses several neuropeptides for the genesis and regulation of physiological and behavioral rhythms. Loss of these peptidergic signals can lead to profound disruption in metabolic homeostasis. Mice lacking the V1a vasopressin receptor show impaired glucose homeostasis and defects in VIP signalling result in overtly altered rhythms of feeding and metabolism (Aoyagi et al., 2007; Bechtold et al., 2008). Moreover, intracranial injection of VIP induces hyperglycemia after treatment with 2-deoxy-D-glucose, an inhibitor of glucose utilization, in both SCN-lesioned rats and sham-operated controls. This finding suggests that VIP acts outside the SCN to elicit hyperglycemia (Chun et al., 1998; Nagai, 2004). More recently icv PACAP and VIP were shown to stimulate hepatic glucose production (Yi et al., 2010). As aforementioned, SCN-mediated activation of PVN neurons results in an activation of the sympathetic input to the liver, ultimately leading to hyperglycemia. Importantly, the hyperglycemia-induced by activation of PVN neurons is basically independent of insulin and corticosterone release, while glucagon release might be involved (Kalsbeek et al., 2004). SCN-mediated rhythmic activation and inhibition of pre-autonomic neurons in the PVN is an important mechanism for the maintenance of a daily rhythm in plasma glucose concentrations (Kalsbeek et al., 2008a). Thus, the observed GRP-induced hyperglycemia might involve an activation of PVN neurons by GRP resulting in increased sympathetic inputs to the pancreas and liver, thus leading to subsequent increased glucagon release and EGP and ultimately hyperglycemia. The GRP content in the rat SCN increases over the course of light period and gradually decreases during the dark period (Shinohara et al., 1993). Moreover, expression of GRP-R and its binding also exhibit a daily rhythm in the SCN, with a peak in the early night (Karatsoreos et al., 2006). Taking together these studies, it might be possible that central administration of GRP at the different time points of the day may have differential effects on glucose metabolism. Since the SCN mediated gradual activation of pre-autonomic neurons in PVN over a day leads to an acrophase of the plasma glucose rhythm toward the beginning of dark period, it is to be expected that the effect of a central GRP infusion might be more robust toward the end of the light period and beginning of the activity period. Furthermore, Li and Rowland (1996) have shown that both peripheral and central administration of Bn causes a strong activation of c-Fos-immunoreactivity (IR) in PVN (Li and Rowland, 1996). Recently, Kallingal and Mintz (2014) showed that microinjection of GRP in the third ventricle induces c-Fos and p-ERK IR in the SCN, PVN and supraoptic nucleus (Kallingal and Mintz, 2014) .Therefore, it would be interesting to know the GRP-R expression rhythm in the ARC and PVN, as these might be the primary targets for the glucoregulatory effects of the ICV infused GRP. Further experiments

aiming at injecting GRP or antagonists specifically in the PVN may help to understand the mechanisms underlying GRP-induced hyperglycemia in more detail. In addition, liver denervation studies in GRP-treated animal may help to further delineate the possible neuronal pathway involved.

In conclusion, we showed a stimulatory effect of central GRP on plasma glucose concentrations. The activation of GRP-R in the brain elevates plasma glucose concentrations by increasing EGP and glucagon release. Since the plasma glucose concentration shows oppositely phased rhythms in nocturnal and diurnal animals and the expression of GRP mRNA in the SCN also shows an anti-phasic rhythm in nocturnal mice and the diurnal grass rat, *Arvicanthis ansorgei* (Dardente et al., 2004), it will be interesting to investigate whether the effects of central GRP on glucose metabolism also depend on the time-of-day and the nocturnal/diurnal nature of a species.

4. Experimental procedures
4.1 Animals

Male Wistar rats (Charles River Breeding Laboratories, Sulzfeld, Germany), weighting 300-350 g, were housed in constant conditions of temperature (21±1 $^{\circ}$C) and humidity 60±5 % with a 12h-light:12h-dark cycle (Lights on at 07:00 h). Animals had *ad libitum* access to food (Irradiated Global 18% protein rodent diet no 2918, Harlan Nederland, Horst, The Netherlands) and water. All experiments were conducted with the approval of the Animal Care Committee of the Royal Netherlands Academy of Art and Science.

4.2 Drugs

Human GRP was obtained from Sigma-Aldich Corp., St. Louis, MO, dissolved in MQ water and stored at -20 $^{\circ}$C. The dose of GRP (0.034 nMol/μl) in the present study was similar to the dose of BN and NB capable to induce hyperglycemia in the study of Plamondon and Merali (1993). MQ or MQ plus GRP were infused via the icv probe at a rate of 1μl/min as a bolus infusion for the first 5 min and 5μl/h for the remaining 155 min.

4.3 Surgical Procedures

After a week of habituation to the NIN animal facility animals were anesthetized by an intramuscular injection of 0.9 ml/kg Hypnorm (Janssen, High Wycombe, Buckinghamshire, UK), followed by a subcutaneous injection of 0.3 ml/kg Dormicum (Roche, Almere, The Netherlands). Silicon catheters were placed into the right jugular vein and left carotid artery for intravenous infusion of the stable glucose isotope and blood sampling, respectively. After the placement of these catheters, vascular lines were closed with a mixture of polyvinylpyruvidon (PVP; Sigma-Aldich Corp., St. Louis, MO), heparin (LEO Pharma,

Ballerup, DK) and amoxicillin (Centrafarm, Etten-Leur, NL). ICV probes were placed in the left cerebral ventricle using a standard Kopf stereotaxic apparatus (anteroposterior: -0.8 mm, lateral: 2.0 mm, ventral: -3.2 mm, angle: 0). Catheters and icv probe were fixed on top of the animals head using dental cement. Probe placement was confirmed by inspecting post-mortem brain slices and only animals showing probe placements in the lateral ventricle were included in the final analysis. These techniques allowed us to conduct experiments in freely moving and awake animals without inflicting stress. Experiments were performed 8-10 days after surgery, only after the recovery of pre-surgical body weight and with animals showing a healthy state.

4.4 Description of the experiment

One day before the actual infusion experiment, animals were hooked up to a metal collar. On the morning of the experimental day, animals were connected to blood sampling and infusion lines, which were attached to the metal collar and kept out of reach from the rats by means of a counterbalanced arm. At the same time (~07:30 AM, i.e., after lights on) food was removed from the cage. This system allowed us to do all subsequent manipulations outside the cage, without having to handle the animals anymore, which very much minimized the stress during blood sampling and drug infusion.

4.5 Endogenous glucose production

To assess endogenous glucose production (EGP), $[6,6-^2H_2]$ glucose was used as a tracer. The intravenous infusion of $[6,6-^2H_2]$ glucose started at t=0 at a rate of 3 ml/min for first 5 min (8 μmol) and 500 μl/hr (16.6 μmol/hr) for remainder of the infusion. Blood samples were taken at t = -5 min for background enrichment (t=0 was at 11:00 AM, i.e., 4 h after lights on), at t=90, t=100, and t=110 min to determine enrichment during the equilibrium state and every 20 min from t=130 min until t=270 to determine enrichment during the experimental process.

ICV infusion of vehicle (MilliQ water at pH 6) or GRP started from t=110 min (~13:00 PM, i.e., 6 h after lights on) until the end of the experiment (0.034 nMol/μl GRP; 1μl/min for the first 5 minutes and 5μl/h for the remainder of the infusion) (**Figure 1**). Immediately after completion of the experiment, animals were sacrificed with a lethal dose of intravenous pentobarbital.

The experiment consisted of two groups: Veh (n=6) and GRP (n=10). Two out of the total of 18 animals operated upon had to be excluded because of technical problems, i.e., a blocked cannula and a pre-mature death of an animal right after the experiment.

4.6 Laboratory method/analysis

During the experiment, glucose concentrations were determined by a glucometer (Abbott). Blood samples were collected in tubes on ice containing heparin and later centrifuged at $+4°C$. Plasma was isolated and stored at $-20°C$ for further analysis.

Plasma concentrations of insulin, glucagon and corticosterone were measured employing radioimmunoassay kits (Millipore, Billerica, USA for insulin and glucagon and MP Biomedicals, Orangeburg, USA for corticosterone). Plasma [6,6-^{2}H$_2$] glucose enrichment was measured by the gas chromatography-mass spectroscopy (GCMS) and EGP was calculated (Steele, 1959; Ackermans et al., 2001). Due to the limited volume of plasma, we selected the t = 100, 150, 190, 230, 270 min and t = 90, 110, 130, 170, 210, 250 min time points for measurement of insulin and glucagon, respectively.

4.7 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by SigmaPlot (version 12, SPSS Inc, Chicago, IL, USA). Significance was defined at $p < 0.05$.

Two-way ANOVA with repeated measures (rmANOVA) were performed to compare glucose, EGP, glucagon, insulin and corticosterone levels. If appropriate, post-hoc analysis was performed using Tukey method.

ACUTE SLEEP RESTRICTION SEVERELY IMPAIRS GLUCOSE TOLERANCE IN RATS

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Abstract

Chronic sleep curtailment in humans has been related to impairment of glucose metabolism. To better understand the underlying mechanisms, the purpose of the present study was to investigate the effect of acute sleep deprivation on glucose tolerance in rats. A group of rats was challenged by 4-h sleep deprivation in the early rest period, leading to prolonged (16 h) wakefulness. Another group of rats was allowed to sleep during the first 4-h of the light period and sleep-deprived in the next 4 h. During treatment, food was withdrawn to avoid a post-meal rise in plasma glucose. An intravenous glucose tolerance test (IVGTT) was performed immediately after the sleep deprivation period. Sleep deprivation at both times of the day similarly impaired glucose tolerance and reduced the early-phase insulin responses to a glucose challenge. Basal concentrations of plasma glucose, insulin and corticosterone remained unchanged after sleep deprivation. Throughout IVGTTs, plasma corticosterone concentrations were not different between the control and sleep-deprived group. Together, these results demonstrate that independent of time-of-day and sleep pressure, short sleep deprivation during the resting phase favours glucose intolerance in rats by attenuating the

first-phase insulin response to a glucose load. In conclusion, this study highlights the acute adverse effects of only a short sleep restriction on glucose homeostasis.

1. Introduction

Recent evidence convincingly shows that sleep is important for metabolic and physiological health. Results from epidemiological studies indicate that short sleep duration for a long period is correlated with obesity and type 2 diabetes (T2D) (Gottlieb et al., 2005; Chaput et al., 2008; Van Cauter and Knutson, 2008; Spiegel et al., 2009; Watanabe et al., 2010). For example, habitual sleep duration of <5-6 h leads to increased body mass index and impaired glucose tolerance or even T2D (Vioque et al., 2000; Chaput et al., 2007; Watanabe et al., 2010). In addition to epidemiological studies that are mainly focused on mild chronic sleep deprivation, laboratory experiments in both human subjects and experimental animals have also linked sleep shortening with metabolic abnormalities in a more acute setting (Spiegel et al., 1999; Barf et al., 2012).

Animal experiments have shown that prolonged sleep deprivation leads to behavioural and physiological changes such as modifications in body temperature, body weight, food consumption and energy expenditure (Rechtschaffen and Bergmann, 1995; Banks and Dinges, 2007; Nedeltcheva et al., 2009a; Vaara et al., 2009; Barf et al., 2012; Markwald et al., 2013). Studies in humans have shown that also the secretion of anabolic (growth hormone, prolactin and testosterone) and catabolic hormones (glucocorticoids and catecholamines) may be affected by sleep disturbances (Nedeltcheva and Scheer, 2014). Moreover, sleep restriction lowers plasma levels of the anorexigenic hormone leptin and elevates those of the orexigenic hormone ghrelin (Spiegel et al., 2004; Taheri et al., 2004; Barf et al., 2012). Both quality and quantity of sleep duration may affect glucose metabolism (Donga et al., 2010; Stamatakis and Punjabi, 2010; Barf et al., 2012). Furthermore, a number of experimental studies with human volunteers suggest that even partial sleep disturbance leads to impaired glucose tolerance and insulin sensitivity, i.e., indicators of a pre-diabetic condition (Spiegel et al., 1999; Tasali et al., 2008; Donga et al., 2010; Schmid et al., 2011; Robertson et al., 2013). Of note, the metabolic profile observed after sleep deprivation shares several similarities with T2D, including decreased muscle glucose uptake, increased liver glucose output and pancreatic β-cell dysfunction (Spiegel et al., 1999; Buxton et al., 2010; Donga et al., 2010; Buxton et al., 2012). Most of the experiments conducted in humans and animals focused on partial or complete sleep restriction during part or the whole resting period. So far no study assessed how acute, short term sleep deprivation affects glucose regulation. Therefore, we aimed to investigate the acute effect of short term sleep deprivation on glucose homeostasis in rats. In order to do so, rats were kept in a light-dark cycle and transferred to constant darkness. On the first day of

constant darkness animals were subjected to an intravenous glucose tolerance test (IVGTT) immediately after a 4-h sleep deprivation period in either the beginning or middle of the rest period.

2. Methods

All the experiments were performed in accordance with the U.S. National Institute Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Directive 2010/63/EU) and approved by the Regional Ethical Committee of Strasbourg for Animal experimentation (CREMEAS) and French Ministry of Higher Education and Research (#01050.01).

2.1 Animals

Male Wistar rats (Janvier Laboratories, France) were maintained at 23°C under a 12 h light/12 h dark cycle (Light intensity during light and dark periods (red light on) was respectively 200 lux and < 3 lux). Lights on at 07:00 AM and lights off at 07:00 PM defined *Zeitgeber* time (ZT) 0 and ZT12, respectively. Animals had *ad libitum* access to food and water and were housed individually in Plexiglas cages (28 × 28 × 40 cm) throughout the experiments. On the day of the experiment, animals were transferred into constant darkness (DD; red light, < 3 lux).

2.2 Experimental design

After a week of habituation, but only when they had reached a body weight of >300 g, animals were implanted with an intra-venous silicone catheter through the right jugular vein, according to the method of Steffens (Steffens, 1969). Two weeks after the surgery, when animals had gained pre-surgery body weight again, all animals were transferred to DD. Rats (n = 6 per group) were either sleep deprived (SD) from circadian time (CT) 0 (defining projected time of lights on during the previous light-dark cycle) to CT4 (for early subjective day sleep deprivation) or allowed to sleep from CT0 to CT4 and sleep deprived from CT4-8 (for middle of subjective day sleep deprivation) by gentle handling or left undisturbed as controls (CTR). Four hours of sleep deprivation by gentle handling is enough to enhance slow-wave sleep during the recovery period in rats (Kostin *et al.*, 2010).An IVGTT was performed immediately after sleep deprivation. Animals were connected to the blood sampling catheter in the last hour of sleep deprivation, which was attached to a metal collar and kept out reach of the rats by using a counterbalanced beam. This system allowed all manipulations to be performed outside the cage without any further handling the animals. During the experiment (including sleep deprivation and blood sampling) no food was in the

cages. A glucose solution (0.5 ml, 500 mg/kg body weight) was injected as a bolus via the jugular vein catheter. First, a blood sample (0.2 ml) was collected (t=0), immediately followed by the glucose injection. Subsequently, blood samples (0.2 ml) were taken at t=5, 10, 20, 40 and 60 min. Samples were used to determine plasma concentrations of glucose, insulin and corticosterone at these time points. The total amount of glucose in plasma and total amount of insulin released after the glucose bolus injection was calculated from the area under the curve (AUC) of every individual animal and averaged for the experimental groups.

2.3 Laboratory method/analysis

During the experiment, blood glucose concentrations were determined by a glucometer (Accu-Check, Roche Diagnostic, Meylan, France). Blood samples were collected in tubes on ice containing heparin and later centrifuged at $+4^{\circ}$C. Plasma was isolated and stored at -20°C for further analysis of insulin and corticosterone. Plasma concentrations of insulin and corticosterone were measured employing radioimmunoassay kits (Millipore, Billerica, USA for insulin and MP Biomedicals, Orangeburg, USA for corticosterone).

2.4 Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by SigmaPlot (version 12, SPSS Inc, Chicago, IL, USA). Significance was defined at p < 0.05. Two-way ANOVAs with repeated measures (rmANOVA) were performed to compare glucose, insulin and corticosterone levels for different samples. Three-way ANOVAs were performed to compare glucose, insulin and corticosterone levels according to sample timing and sleep status at the two CTs. Two-way ANOVAs were performed to compare basal glucose, insulin, corticosterone, AUCs and I/G_{5-0} between the experimental groups at the two CTs. If appropriate, post-hoc analysis was performed using Tukey's test.

3. Results

IVGTTs were performed immediately after the sleep deprivation (SD), during the beginning as well as in the middle of the subjective day as described in experimental protocol outline (**Figure 1**). SD in both early and mid-subjective day caused an impaired glucose tolerance. Injection of the glucose bolus resulted in an immediate and pronounced increase in plasma concentrations of glucose and insulin in both control and sleep deprived animals (**Figure 2 A, B, D, E**). Highest glucose concentrations were detected 5 min after the bolus injection, directly followed by a rapid decrease. Within 20 min after injection, glucose concentrations had returned to pre-infusion concentrations again. Both during early and mid-subjective day, ANOVA showed significant effects of *SD* ($F_{1,50} = 6.42$, P = 0.03 and $F_{1,50} = 13.42$, P = 0.004),

Sample timing ($F_{5,50} = 34.85$, $P < 0.001$ and $F_{5,50} = 35.63$, $P < 0.001$) and *Interaction* ($F_{5,50} = 5.56$, $P < 0.001$ and $F_{5,50} = 12.31$, $P < 0.001$). Post-hoc analysis revealed that plasma glucose levels were significantly elevated at t=5 min in sleep deprived compared to control animals at both CT4 and CT8 ($P < 0.001$). The 3-way ANOVA showed no significant effects of *Time-of-day* ($P = 0.194$), or the interaction of *Sample timing * Time-of-day* ($P = 0.885$), *SD * Time-of-day* ($P = 0.512$) or *Sample timing * SD * Time-of-day* ($P = 0.587$), indicating that the glucose responses at both time points were very similar.

Plasma insulin levels also increased in response to the glucose bolus in both the sleep deprived and control groups, at t = 5 min. ANOVA showed no significant effect of *SD* during either early or mid-subjective day ($F_{1,50} = 0.73$, $P = 0.41$ and $F_{1,50} = 0.25$, $P = 0.62$), but *Sample timing* ($F_{5,50} = 18.72$, $P < 0.001$ and $F_{5,50} = 2.44$, $P = 0.046$) and *Interaction* ($F_{5,50} = 2.89$, $P = 0.02$ and $F_{5,50} = 4.46$, $P = 0.002$) did show significant effects at both time points. Post-hoc analysis revealed that insulin levels were significantly higher in the sleep deprived group at t = 10 min during both the beginning and middle of the rest period ($P = 0.025$ and $P = 0.004$). In addition, at t = 40 plasma insulin levels were increased in the control group (CT 4-8) ($P = 0.02$). The 3-way ANOVA showed significant effects of *Sample timing * Time-of-day* ($P = 0.035$) and *Sample timing * SD * Time-of-day* ($P = 0.012$), but not *Time-of-day* ($P = 0.838$) or *SD * Time-of-day* ($P = 0.392$) interactions, indicating small time-course differences of insulin responses during IVGTT at CT4 and CT8 (see **Figure 2 B, E**).

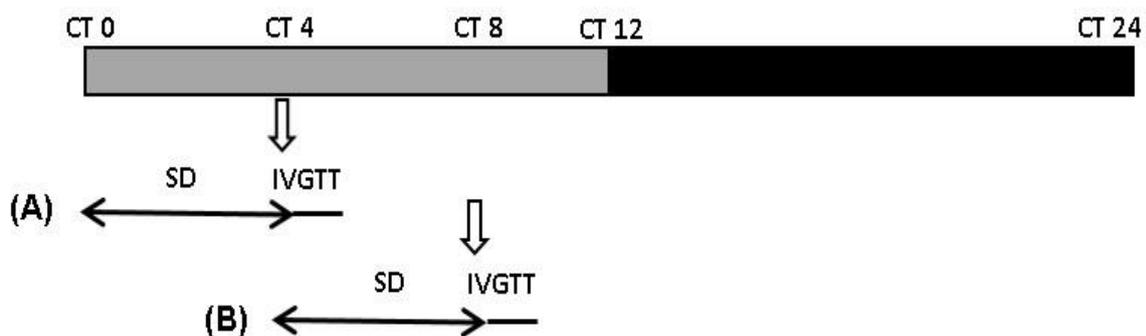**Figure 1 Experimental protocol showing the time line of sleep deprivation and intravenous glucose tolerance tests (IVGTTs). (A) Sleep deprivation in early rest period between Circadian Time (CT) 0 to CT4 and IVGTT from CT4 to CT5. (B) Sleep deprivation in middle of rest period between CT4 to CT8 and IVGTT from CT8 to CT9. SD = sleep deprivation; Gray area: subjective day; Black area: subjective night.**
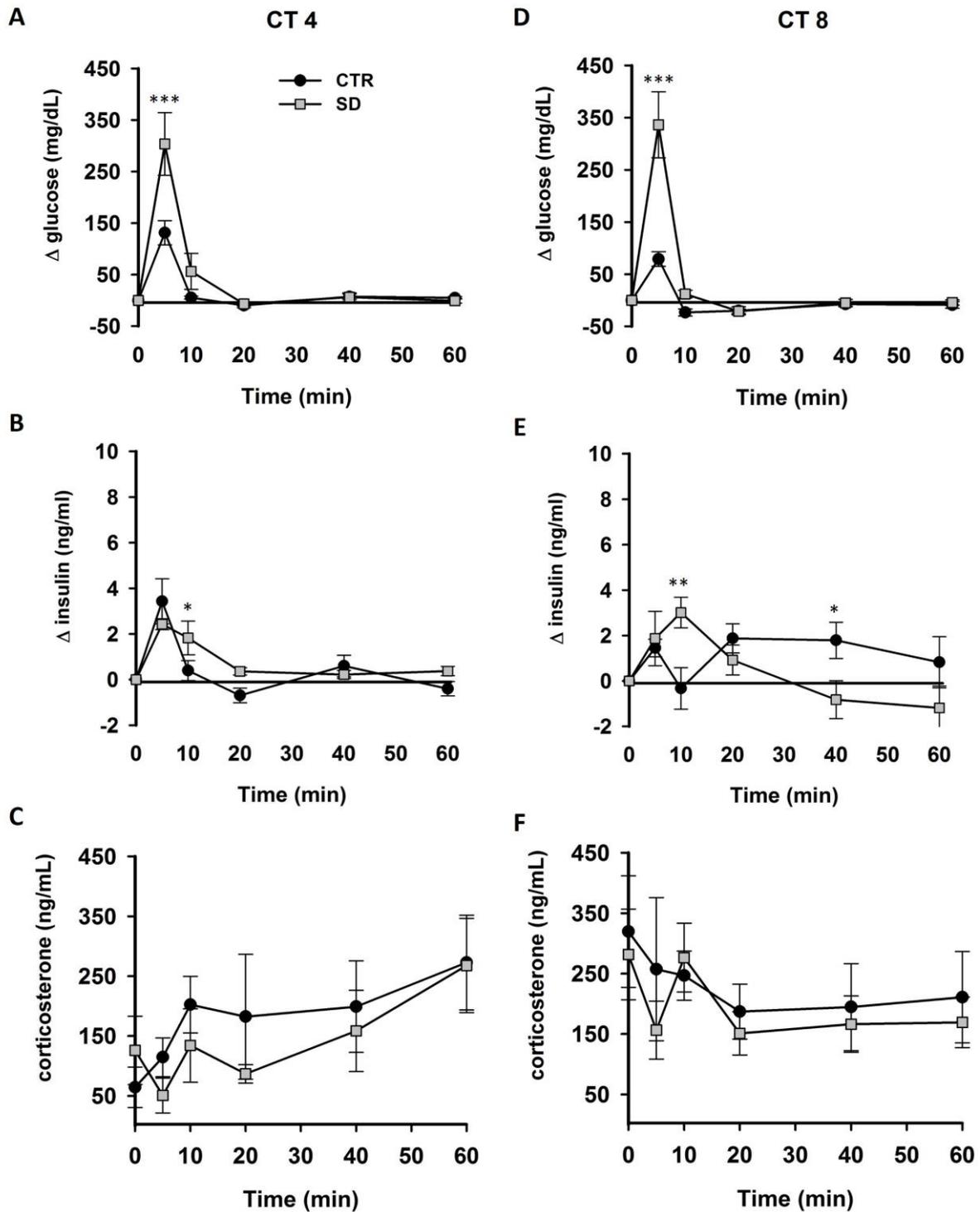
Figure 2 Intravenous glucose tolerance tests (IVGTTs) in rats after sleep deprivation. Relative changes in plasma glucose concentration (A, D), relative change in plasma insulin concentration (B, E) and plasma corticosterone concentration (C, F) after a glucose bolus (500 mgkg^{-1} intravenous) during IVGTTs starting at Circadian Time (CT) 4 and CT8. Black circles: control animals; gray squares: sleep deprived animals. CTR = control, SD = sleep deprived. All groups n = 6. Data are presented as mean ± SEM. * $P <$ 0.05, ** $P <$ 0.005, *** $P <$ 0.001.

To test the possibility of activation of the hypothalamo-pituitary-adrenal (HPA)-axis due to sleep deprivation and intervention of IVGTTs, we measured corticosterone levels before and during IVGTTs. Basal levels of plasma corticosterone were not affected by sleep deprivation ($F_{1,20}$ = 0.03, P = 0.8), but basal levels were higher at CT 8 than at CT 4 ($F_{1,20}$ = 9.1, P = 0.007) (**Figure 3 C**). During IVGTTs, ANOVA showed no significant effect of SD during either early or mid-subjective day ($F_{1,50}$ = 0.67, P = 0.43 and $F_{1,50}$ = 0.38, P =0.54) (**Figure 2 C, F**). The 3-way ANOVA did not show significant effects of *SD * Time-of-day* (P = 0.99), *SD * Sample timing* (P = 0.92) and *Sample timing * SD * Time-of-day* (P = 0.9), but it detected an effect of *Time-of-day* (P = 0.01) and *Sample timing * Time-of-day* (P = 0.04), indicating the higher mean corticosterone levels during the CT4-8 IVGTT.

Basal levels of plasma glucose did not change due to sleep deprivation ($F_{1,20}$ = 2.54, P = 0.12), though the effect of time-of-day on plasma glucose concentration was apparent ($F_{1,20}$ = 10.33, P = 0.004), with higher levels later in the day (**Figure 3 A**). Like basal glucose, basal plasma concentrations of insulin also depended on the time-of-day ($F_{1,20}$ = 7.55, P = 0.012) (**Figure 3 B**). Post-hoc analysis showed that especially in the SD group basal insulin was higher at CT8 compared to CT4 (P = 0.017).

To estimate the ability of the β-cells to respond to a glucose challenge, we calculated insulin secretion over the first 5 min after the injection ($\Delta I5$-0) divided by the difference between the glucose concentrations during the same time period ($\Delta G5$-0), i.e., I/G_{5-0}. ANOVA showed significant effects of *SD* ($F_{1,20}$ = 12.91, P = 0.002) and *Time-of-day* ($F_{1,20}$ = 6.40, P = 0.02) (**Figure 3 D**), with I/G_{5-0} being lower at CT8. Post-hoc analysis revealed that SD significantly decreased the I/G_{5-0} in both early and late subjective day (P = 0.008 and P = 0.044).

We also analysed the AUCs as an estimation for the amount of glucose and insulin released after the bolus injection of glucose (**Figure 3 E, F**). ANOVA showed that SD significantly affected the AUC of glucose ($F_{1,20}$ = 17.77, P < 0.001). Post-hoc analysis revealed that SD significantly increased the glucose AUC in early and middle of subjective day (P = 0.01 and P = 0.006). On the other hand, insulin AUCs were not significantly affected by *SD* ($F_{1,20}$ = 1.18, P = 0.29) and *Time-of-day* ($F_{1,20}$ = 0.64, P = 0.43).
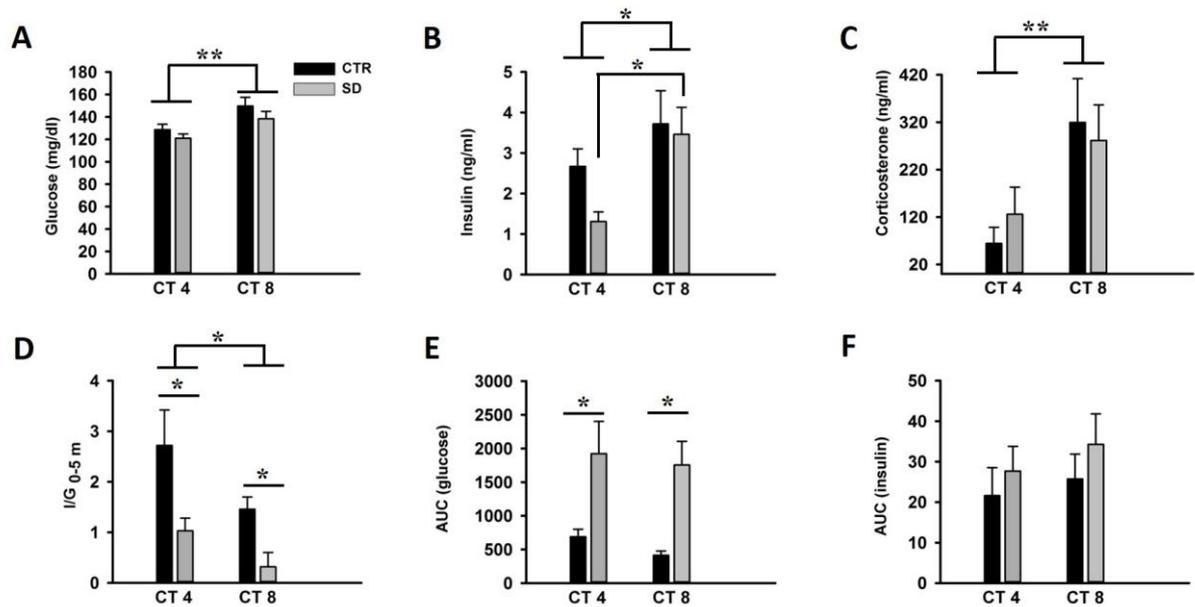
Figure 3 Basal glucose and hormone concentrations during intravenous glucose tolerance tests (IVGTTs) in rats after sleep deprivation (all groups n = 6). (A) Basal plasma glucose concentrations were significantly higher at Circadian Time (CT) 8 compared to CT 4. (B) Basal plasma insulin concentrations were significantly higher at CT8 in the SD group. (C) Basal plasma corticosterone concentrations were significantly higher at CT8 compared to CT4. (D) I/G$_{t0-5}$: the ratio of ΔI5-0 to ΔG5-0 (ΔI5-0/ΔG5-0 as a measure of the insulin response to glucose in the first 5 min) was reduced significantly after sleep deprivation in the early and late rest period. (E) AUC of the plasma glucose response and (F) the plasma insulin response after a glucose bolus at CT4 and CT8 after a 4-h sleep deprivation. Black bars: control group; gray bars: sleep deprived group. CTR = control, SD = sleep deprived, AUC= area under the curve. AUC was calculated from t=0 till t=20 min. Data are presented as mean ± SEM. * $P < 0.05$, ** $P < 0.005$.

4. Discussion

There is increasing evidence from human and animal studies that disturbed sleep is associated with perturbations in glucose homeostasis (Spiegel et al., 1999; Barf et al., 2010). It is not clear, however, how acute sleep deprivation in terms of duration and timing during the rest period impacts on glucose metabolism. In the present study, we show that in rats a short period (4 h) of sleep deprivation is sufficient to severely impair glucose tolerance and reduce the early-phase insulin response to an intravenous glucose load.

4.1 Methodological considerations

The detrimental impact on glucose metabolism of short sleep duration over many days together with misaligned or irregular sleep has been reported in several studies (Briancon-Marjollet et al., 2015). A few studies also investigated the effects of acute sleep restriction (i.e., within one circadian cycle) on glucose homeostasis in humans (Schmid et al., 2009; Donga et al., 2010) and rats (Barf et al., 2010). In both cases, the effects of sleep restriction

were tested at only 1 time point. In both humans and rats glucose homeostasis is strongly influenced by time of day (Kumar Jha et al., 2015). Among others, glucose tolerance improves from the beginning of the rest period to the onset of the activity onset (la Fleur et al., 2001a). Such daily variations may thus modulate the effects of sleep deprivation on glucose metabolism. Therefore, we set out to investigate whether the effects of sleep deprivation are influenced by time of day.

For sleep deprivation during early daytime, rats were forced to be awake during the first 4 h of the usual resting period (CT0-4), thus prolonging the period of wakefulness from about 12 h to 16 h. For sleep deprivation later during the light period, there are two options: either keeping the rats awake during a longer time span (e.g., 20 h) or allowing sleep during the early part of the rest period followed by sleep restriction during the latter part of the rest period. We chose the latter option as it permits to test the effect of a similar period of sleep deprivation (i.e., 4 h), but occurring at a different time of day. Notwithstanding, the fact that with sleep deprivation during the latter part of the light period sleep propensity was probably decreased compared to the rats sleep deprived in early morning, glucose tolerance was similarly altered in rats sleep deprived in either the early or middle part of the rest period. Thus, independently of time-of-day and sleep pressure, sleep restriction is capable of altering glucose homeostasis. In fact, the adverse effect of sleep deprivation on glucose tolerance was much stronger than the diurnal variation in glucose tolerance. Thus, the effect of sleep deprivation completely overruled the improvement of glucose tolerance during the light period as seen in the control animals.

In human studies that investigated the effects of acute sleep restriction (Schmid et al., 2009; Donga et al., 2010), lights were on during sleep restriction, which could stimulate wakefulness and inhibit melatonin secretion (Redlin, 2001; Chellappa et al., 2011). In rats light exposure has been reported to stimulate glucocorticoid release (Buijs et al., 1999) and increase plasma glucose (Challet et al., 2004). Thus, to avoid interferences with the outcomes studied, lights were turned off during the present experiment. Moreover, to rule out any putative bias due to changes in food intake of sleep deprived rats, food was removed before the start of sleep deprivation and blood sampling.

Several procedures have previously been used to induce sleep deprivation in rodents, including forced locomotion, gentle handling, and short platform-over-water. Gentle handling has the advantage to avoid the confounding effect of hyperactivity triggered by forced locomotion. In addition, it is thought to prevent the stressful effects of platform-over-water and forced locomotion. Our assumption that gentle handling is suitable for short periods of

sleep deprivation is supported by finding similar levels of basal blood corticosterone in control and sleep deprived rats, indicating that the experimental groups were not stressed by gentle handling.

4.2 Glucose tolerance and hormonal changes

In our study, a single period of 4 h of sleep deprivation either in early or middle of the light period did not modify the basal levels of plasma glucose, insulin and corticosterone, a finding consistent with the lack of significant effect of a single night limited to 4.5 h sleep in human subjects (Schmid et al., 2009). The data from the IVGTT show that sleep deprivation in rats strongly reduces glucose tolerance, as evidenced by the rise in plasma glucose concentrations to higher levels and for a longer time. Several effects may participate in the reduced glucose tolerance. First, although the total amount of insulin released in the sleep deprived group was not changed (**Figure 3 F**), the reduced early insulin responses at both time points investigated indicates a reduced or at least inadequate sensitivity of the beta cells. This finding is consistent with a previous rat study by Barf and colleagues using a much longer period of sleep deprivation (i.e., 20 h) (Barf et al., 2010).

The decreased glucose tolerance in sleep deprived animals during IVGTT may results from either higher glucose production or less glucose uptake. The data from the present study could not differentiate whether the hyperglycemia is due to reduced glucose uptake or more glucose production. The reduced early insulin response in the sleep deprived groups will result both in a reduced glucose uptake as well as a lesser inhibition of glucose production. In order to understand further the mechanism of hyperglycemia, experiments using the stable isotope dilution technique to determine endogenous glucose production (EGP) need to be done. Sleep deprivation might trigger glucagon release, which would subsequently result in a higher EGP. Although at a first glance, this hypothesis appears unlikely because acute sleep deprivation has an inhibitory effect on circulating glucagon levels in humans (Schmid et al., 2009), further assays of plasma glucagon are needed to evaluate this possibility.

An alternative explanation for the increased glucose levels during the IVGTT could be an increased activity of the HPA-axis, as a consequence of stress during acute sleep deprivation. In humans, most studies reported no acute change in glucocorticoid levels after sleep deprivation (Everson and Crowley, 2004; Donga et al., 2010), although delayed effects (i.e., the day after) have been reported (Leproult et al., 1997). By contrast, depending on the procedure of sleep deprivation in animal studies, sleep disturbances can increase glucocorticoid release (Baud et al., 2013). However, Barf et al (2010) reported no differences in plasma corticosterone levels between sleep deprived and control rats (Barf et al., 2010). In

the present study, basal levels of plasma corticosterone and corticosterone release during IVGTTs were not different in sleep deprived rats as compared to undisturbed controls, ruling out the possibility of major acute activation of the adrenal via the HPA- or sympatho-adrenal axis.

4.3 Possible mechanisms

Our results revealed an altered insulin response to the glucose load during the first 5 min in sleep deprived animals. The diminished early-phase response of insulin after sleep deprivation suggests a reduced or impaired sensitivity of the beta cells to a glucose challenge. This defect may depend upon disturbances in the sensitivity of the pancreatic beta cells to glucose and/or its control by the autonomic nervous system. The latter possibility is supported by the fact that sleep deprivation results in sympathetic activation and release of catecholamines in the general circulation (Levy et al., 2009). Hyperactivity of the sympathetic branch of the autonomic nervous system may lead to insulin resistance (Egan, 2003). Thus, the reduction in the early-phase insulin response to glucose might be related to an increased sympathetic and/or decreased parasympathetic activity. Moreover, increased activity of the sympathetic nervous system would also stimulate glucose production. Future work should determine possible changes in the sympathovagal balance under the present conditions of sleep deprivation.

Considering that some actions of sleep deprivation on peripheral functions may result from sympathetic activation, what could be the central structures mediating these effects? A likely candidate is the hypothalamic orexin system, because this neuropeptide is involved not only in the regulation of the sleep/wake cycle, but also in the daily rhythm of glucose metabolism (Sakurai, 2007; Kalsbeek et al., 2010a) . Activity of orexin neurons in the perifornical region of the hypothalamus is highest during the wake period and during sleep deprivation (Estabrooke et al., 2001). These orexin neurons also participate in the control of endogenous glucose production in the liver via the autonomic nervous system (Yi et al., 2009). Furthermore, orexin appears to regulate insulin sensitivity, because mice lacking orexin show an age-related development of systemic insulin resistance (Hara et al., 2005; Tsuneki et al., 2008). At the same time, orexin-deficient narcoleptic patients with cataplexy show body mass index-dependent metabolic alterations, including insulin resistance (Poli et al., 2009). Finally, Tsuneki et al, (2015) have shown that orexin has bi-directional effects on hepatic gluconeogenesis via the autonomic nervous system (Tsuneki et al., 2015). To test whether orexin neurons are involved in the autonomic control of hepatic glucose production and/or

pancreatic sensitivity to glucose, orexin-antagonist and organ-specific denervation studies should be performed during sleep deprivation.

Like orexin, also the serotonin system is involved in arousal and the regulation of glucose metabolism (Asikainen et al., 1997; Versteeg et al., 2015). Injection of serotonin leads to hypoglycaemia in rats and mice (Yamada et al., 1989; Sugimoto et al., 1990). Mice deficient in serotonin reuptake transporters and the 5-HT_{2c} receptor in pro-opiomelanocortin neurons of the arcuate nucleus in the hypothalamus show impaired glucose metabolism (Xu et al., 2010; Chen et al., 2012). Of note, serotonin levels in the central biological clock, located in the hypothalamic suprachiasmatic nuclei (SCN), show a daily rhythm in both nocturnal and diurnal rodents (Poncet et al., 1993; Cuesta et al., 2008). In Syrian hamsters the seasonal changes in metabolic activity result in a seasonal fluctuation from a eu-insulinemic and glucose tolerant state to a hyperinsulinemic and glucose intolerant state. The daily rhythm of SCN serotonin was shown to be severely impaired in glucose intolerant hamsters, indicating a functional link between the SCN, serotonin and glucose metabolism (Luo et al., 1999). However, little is known regarding the effects of sleep deprivation on the hypothalamic serotonin system, or the different serotonin receptor subtypes. Therefore, additional experiments are needed before the hypothalamic serotonin system can be implicated in the sleep deprivation-induced changes in glucose metabolism.

NPY is another hypothalamic neuropeptide involved in the control of feeding, arousal and glucose metabolism (Szentirmai and Krueger, 2006; Kalsbeek et al., 2010c; Wiater et al., 2011). Central administration of NPY results in an increase in EGP in rats, probably by increasing hepatic glucose production (Kalsbeek et al., 2010c). It has been shown by Van Den Hoek et al. (2008) that i.c.v administration of NPY causes insulin resistance via activation of sympathetic output to the liver (van den Hoek et al., 2008). NPY-containing neurons in the arcuate nucleus also project to the paraventricular nucleus of the hypothalamus (PVN), which is a relay centre for the hypothalamic integration of glucose metabolism. Therefore, the presently observed impaired glucose tolerance could have been mediated through an enhanced stimulation of NPY receptors in the hypothalamus. Indeed, in chronic sleep-deprivation studies have shown increases expression of hypothalamic NPY (Koban et al., 2006; Martins et al., 2010). On the other hand, central administration of NPY has been reported to induce wakefulness (Szentirmai and Krueger, 2006).

4.4 Biomedical perspectives

The present study investigated the acute effects of sleep deprivation on glucose homeostasis in rats. Our data show that disturbance of the sleep-wake rhythm during early or late

subjective day by short sleep deprivation acutely affects glucose metabolism by severely impairing glucose tolerance. Our results show that prolonged wakefulness (sleep deprivation during the early resting period) and short duration sleep deprivation (sleep deprivation in the middle of the rest period) impair glucose tolerance to the same extent.

The sleep-wake cycle is oppositely phased in nocturnal and diurnal species according to the astronomical light/dark cycle, while plasma glucose concentrations also show oppositely phased rhythms between nocturnal and diurnal rodents (Dardente et al., 2004). Therefore, it would be interesting to determine whether acute sleep deprivation during the resting period induces the same alterations of glucose metabolism in a diurnal rodent, i.e., being active during the light period as are humans.

Unravelling the mechanisms that underlie the deleterious effects of sleep deprivation on glucose metabolism in rats under tightly controlled conditions may be ultimately relevant for applications in humans.

Acknowledgements

Sleep deprivation and caffeine treatment potentiate photic resetting of circadian clock in a diurnal rodent, the Sudanian grass rat (*Arvicanthis ansorgei*)

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Abstract

Circadian rhythms in nocturnal and diurnal mammals are primarily synchronized to local time by the light-dark cycle. However, non-photic factors, such as behavioural arousal and metabolic cues, can also phase-shift the master clock in the suprachiasmatic nuclei (SCN) and/or reduce the synchronizing effects of light in nocturnal rodents. In diurnal rodents the role of arousal or insufficient sleep in these functions is still poorly understood. In the present study, we examined the effects of behavioural arousal (sleep deprivation by gentle handling or caffeine treatment) on the circadian pacemaker and its responses to light in the diurnal Sudanian grass rat, *Arvicanthis ansorgei*. Phase-shifts of locomotor activity were analysed in grass rats transferred from a light-dark cycle to constant darkness and aroused in early night (sleep deprivation from Circadian time 12 (CT12) to CT16 or caffeine injection at CT14) or late-night (sleep deprivation from CT16 to CT20 or caffeine injection at CT18). Early, but not late-night sleep deprivation induced a significant phase-shift. Caffeine on its own induced no phase-shifts. Both sleep deprivation and caffeine treatment potentiated light-induced phase-delays and phase-advances in response to a 30-min light pulse at CT16 and CT20, respectively. Sleep deprivation in early but not late night potentiated light-induced c-FOS expression in the ventral SCN. Caffeine treatment in midnight triggered c-FOS expression in dorsal SCN. Both sleep deprivation and caffeine treatment potentiated light-induced c-FOS expression in calbindin-containing cells in the ventral SCN in early and/or mid night. These

findings indicate that in contrast to nocturnal rodents in diurnal rodents behavioural arousal during sleep, induced either by sleep deprivation or caffeine, potentiates light resetting of the master circadian clock, in part by activating calbindin-containing cells in the SCN.

Key words: Behavioral arousal, Phase-shift, Phase-response curve, Suprachiasmatic nucleus

1. Introduction

The phasing of the cycle of sleep and arousal with respect to day and night is one of the key rhythmic components that divide animals into night-active (nocturnal) and day-active (diurnal) species. There has been considerable research on circadian rhythms in nocturnal rodents with regard to the synchronizing properties of light and other cues, including behavioural arousal and metabolic factors. Diurnal species, however, have been studied much less as compared to nocturnal rodents, especially for non-photic synchronizing factors. Since with respect to the light-dark cycle the temporal organization of sleep and arousal is inverted between nocturnal and diurnal animals, it is important to determine whether arousal is a synchronizing signal in diurnal animals during the same astronomical period as in nocturnal animals (i.e., daytime) or during the same period of the sleep/wake cycle (i.e., sleep).

In addition to the sleep-wake cycle, a number of other behavioural and metabolic rhythms are oppositely phased relative to the light-dark cycle between nocturnal and diurnal mammals (Kumar Jha et al., 2015). However, the central clock in the suprachiasmatic nucleus (SCN) of the hypothalamus, which regulates rhythmicity of most behavioral and physiological parameter, is most active at the same astronomical time in these two categories of mammals. For instance, glucose utilization and firing rate in the SCN are high during the day and low at night in both diurnal and nocturnal animals (Smale et al., 2003). Genesis of circadian rhythmicity is based on the rhythmic expression of core clock genes such as *Pers* (*Per1-3*), *Crys* (*Cry1-2*), *Bmal1*, *Clock* and nuclear receptors *Rev-erbα/β* and *Rorα/β* (Mohawk et al., 2012). Temporal patterns of expression of most of these clock genes in the SCN are similar in nocturnal and diurnal species (Albrecht et al., 1997; Shearman et al., 2000; Mrosovsky et al., 2001; Caldelas et al., 2003).

The molecular clock machinery is reset by environmental cues. In particular, light can synchronize the SCN neurons via a cascade of transcriptional activation. Photic resetting is mostly mediated by a direct pathway from the retina to the SCN that originates from the photosensitive retinal ganglion cells and releases glutamate and PACAP (Moore and Lenn, 1972; Hattar et al., 2002; Hughes and Piggins, 2012). Both diurnal and nocturnal species are most sensitive to the synchronizing effects of light at night. Within nocturnal and diurnal

species the mechanisms underlying photic resetting are essentially similar in terms of temporal sensitivity and direction of light-induced phase-shifts (Challet, 2007). As a rule, light exposure during the early and late subjective night elicits phase-delays and advances, respectively (Daan and Pittendrigh, 1976; Slotten et al., 2005). In addition to light, numerous non-photic factors have abilities to reset the phase and period of the SCN clock and influence its photic resetting (Mrosovsky, 1996; Challet and Pevet, 2003; Challet, 2007). Some of these factors are based on involuntary arousal in the resting period. Notably, it has been found that sleep deprivation by gentle handling in the nocturnal Syrian hamster during the resting period led to phase-advances of locomotor activity rhythm. Moreover, this treatment attenuates light-induced phase-delays in both hamsters and mice (Mistlberger et al., 1997; Challet et al., 2001; van Diepen et al., 2014). On the other hand, systemic administration of the adenosine antagonist caffeine during the usual resting period induces arousal without shifting the SCN clock in Syrian hamster, but it does blocks light-induced phase-shifts (Antle et al., 2001; Vivanco et al., 2013).

All studies so far concerning non-photic entrainment have been carried out in nocturnal hamsters and mice. To study behavioral arousal as a possible non-photic entraining agent in diurnal rodents, we first examined whether sleep deprivation or caffeine-induced arousal affects the phase-shifting effect of light in the Sudanian grass rat (*Arvicanthis ansorgei*). Second, since c-FOS induction has been used as a functional marker to investigate the molecular mechanisms of entrainment, we also examined c-FOS expression in the SCN of behaviorally aroused animals. Third, we pheno-typed the neurons that show c-FOS expression in the SCN. Fourth, to better understand the neurochemical basis and assess the state of arousal, we measured serotonin, adenosine and caffeine concentrations in the SCN and midbrain raphe area of animals, aroused or not by caffeine and sleep deprivation.

2. Materials and Methods

All the experiments were performed in accordance with the U.S National Institute Health Guide for the Care and Use of Laboratory Animals (1996), the French National Law (implementing the European Directive 2010/63/EU) and approved by the Regional Ethical Committee of Strasbourg for Animal experimentation (CREMEAS) and French Ministry of Higher Education and Research, #01050.02).

2.1. Animals and housing conditions

Male Sudanian grass rats (*Arvicanthis ansorgei*), 3-4 months old, were obtained from our breeding colony (Chronobiotron platform, UMS3415, CNRS, University of Strasbourg) in

Strasbourg, weighing 100–150 g at the beginning of the study. During baseline, animals were maintained at 23°C under a 12 h light/12 h dark cycle (LD) (light intensity during light and dark periods (red light on) was respectively 200 lux and < 3 lux). Lights went on at 07:00 AM and off at 07:00 PM defining *Zeitgeber* time (ZT) 0 and ZT12, respectively. During the experiments, animals were housed individually in Plexiglas cages (35 × 22 × 40 cm) equipped with running wheels (diameter: 34 cm) with *ad libitum* food and water in LD. After a period of 2 weeks, animals were transferred into constant darkness (DD; red light, < 3 lux). Animals included in the present study displayed bimodal patterns of diurnal locomotor activity.

2.2. Experimental design

Sleep deprivation

Experiment 1: Early night sleep deprivation

We first studied the circadian clock resetting by sleep deprivation (SD) in early night. On the first day of DD, 24 animals were transferred to new cages (without running wheel) at projected Circadian Time (CT) 12 (defining projected time of lights off during the previous light-dark cycle). Twelve out of them were sleep deprived by gentle handling (Hubbard et al., 2015) between CT12 to CT16. The remaining 12 animals were kept undisturbed as controls (CTR). After sleep deprivation, half of the animals from both the sleep deprived and control group were subjected to a 30-min light pulse of fluorescent white light (200 lux at the level of animals; "Light") at CT16, while the other animals were kept in darkness ("Dark"). Thereafter, all animals were transferred back to their home cages. Thus, there were 4 groups (each n=6): (i) CTR + Dark, (ii) SD + Dark, (iii) CTR + Light and (iv) SD + Light.

To investigate the molecular responses, the 24 animals that were used in the above mentioned behavioural experiment were divided into four groups again (n = 6 per group, as indicated above, but newly randomized) and transferred to DD where they were sleep deprived and exposed to a light pulse as described above. Animals were euthanized under a dim red light (TL-D 18W Red SLV, Philips, <3 lux at the level of animals) 1 h after the end of sleep deprivation in the "Dark" groups and 30 min after light exposure in the "Light" groups, i.e., CT17.

Experiment 2: Late night sleep deprivation

The procedures for this study were the same as for Experiment-1, except that the sleep deprivation period was from CT16 to CT20, while animals from group (iii) and (iv) were exposed to a light pulse at CT20. Animals were euthanized at CT21.

Caffeine treatments

Experiment 3: Morning caffeine treatments

Treatments were administered on the first day of DD. Each animal received an intraperitoneal (i.p) injection of caffeine (CAF) (30 mg/kg in 0.9% saline; C0750, Sigma-Aldrich, St. Louis, MO, USA) or vehicle (0.9% saline; VEH) at CT0 followed 2 h after the injection by a 30-min light pulse ("Light") or no light ("Dark") at CT 2. Thus, there were 4 groups (n=6): (i) VEH + Dark, (ii) CAF + Dark, (iii) VEH + Light and (iv) CAF + Light.

Experiment 4: Midday caffeine treatments

The procedure for this treatment was the same as for Experiment-3, except that the timing of CAF or VEH injection was at CT6 and that animals of group (iii) and (iv) were exposed to a light pulse at CT8.

Experiment 5: Early night caffeine treatments

The procedure for this treatment was the same as for Experiments-3 and -4, except that the timing of the CAF and VEH injection was at CT14 and animals of groups (iii) and (iv) were exposed to light pulses at CT16.

To investigate the molecular responses, the 24 animals that were used in the above mentioned behavioural experiment were divided into four groups again (n = 6 per group, as indicated above, but newly randomized) and transferred to DD. Animals were treated with CAF or VEH at CT14, in addition, animals in groups (iii) and (iv) were exposed to a light pulse at CT16. Animals were euthanized under a dim red light (TL-D 18W Red SLV, Philips, <3 lux at the level of animals) 3 h after CAF treatment i.e. CT17.

Experiment 6: Midnight caffeine treatments

The procedure for this treatment was the same as for Experiment-5, except that the timing of the CAF and VEH injection was at CT18 and animals of groups (iii) and (iv) were exposed to a light pulse at CT20 and animals were euthanized at CT 21.

2.3. Recording and analysis of behavioural data

Wheel-running activity was continuously recorded and stored in 5 min bins by Vital-View (Minimitter Inc., Sunriver, OR, USA). All parameters for wheel-running activity were determined with Clocklab Software (Actimetrics, Evanston, IL, USA). Beginning and end of activity at ~ZT/CT0 and ~ZT/CT12 were considered as onset and offset respectively. Throughout the experiments, we selected offset points (~ZT12/CT12) for calculation of phase-shifts. The magnitude and the direction of elicited phase-shifts were calculated by linear regression (Clocklab) encompassing the 6 days before the sleep deprivation or caffeine treatments and the 6 days after it, excluding transitory cycles. Phase-shift magnitudes were

calculated by considering the interval between the two fitted lines by a researcher blind to experimental procedures. Phase-shifts in response to sleep deprivation, caffeine treatment, light plus sleep deprivation or caffeine treatment plus light were assessed using the Aschoff Type II procedure (Refinetti, 2006).

2.4. Immunohistochemistry

Animals for immunocytochemistry were deeply anesthetized with a high dose of sodium pentobarbital (i.p. 150 mg/kg) and intracardially perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

Each brain was rapidly removed and post-fixed in the same fixative for 24 hrs, than cryoprotected in 30% sucrose for 48 h. Brains were frozen in isopentane at -40°C and stored in -80°C. Coronal sections of 30 μm through the SCN were prepared with a cryostat (Leica CM 3000) and stored at -20°C in Watson solution.

Single labelling for Fos within the suprachiasmatic nucleus

For c-FOS immunostaining, free-floating brain sections were washed in phosphate buffer saline (PBS), pH 7.4 and incubated in a solution of 3% H2O2 (30% Hydrogen peroxide, Sigma) in PBS for 30 min. Brain sections were rinsed in PBS for 10 min three times and (i) incubated in 10% normal goat serum (Chemicon®, Merck-millipore, USA) in PBS with 0.3% Tween-20 (Sigma) for 1 h, followed by (ii) an incubation with a rabbit polyclonal anti-c-FOS antibody (1:4000, SC-52, Santa Cruz Biotechnology, CA, USA) for 24 h at 4°C, followed by (iii) biotinylated secondary antibody for 2 h at room temperature (1:500, goat anti-rabbit; Vector Laboratories; in PBS with 0.3% Tween-T20), followed by (iv) avidin-biotin complex (ABC) for 1 h at room temperature (0.2% each avidin and biotin solution; ABC Vectastain Kit; Vector laboratories; in PBS with 0.05% Tween-20). Sections were then reacted in 3,3 diaminobenzidine tetrahydrochloride (DAB; 0.5 mg/ml, Sigma) with 0.015% H_2O_2 in tap water. In between each step after (ii), brain sections were rinsed three times for 10 min in PBS. All tissue sections were mounted onto gelatin-coated slides, dehydrated through a series of alcohol, soaked in toluene and coverslipped with Eukitt (Chem lab, Zedelgem, Belgium)

Double labelling for Fos and calbindin within the suprachiasmatic nucleus

To phenotype the Fos immunoreactive neurons in the SCN, we performed double labelling of Fos and calbindin. Initially brain sections were processed for c-FOS using a nickel enhanced DAB procedure as described in the single labelling for c-FOS. After reaction with ABC, tissue was reacted by mixing DAB (0.5 mg/ml), 0.015% H_2O_2 and 2.5% nickel sulphate. Following the Fos reaction, the tissue was rinsed and processed for detection of calbindin-immunoreactivity as follow: brain sections were incubated with a mouse monoclonal anti-

calbindin-D-28k antibody (1:2000, C9848, Sigma-Aldrich, St. Louis, MO, USA) for 24 h at room temperature, followed by the same procedures as reported above for c-FOS single labelling (i.e., biotinylated secondary antibody, ABC, and incubation with DAB without nickel sulphate). All the tissue sections were mounted onto gelatin-coated slides, dehydrated through a series of alcohol, soaked in toluene and coverslipped with Eukitt (Chem lab, Zedelgem, Belgium).

2.5. Microscopic quantification

A single section through the central SCN was selected for quantification. Cells were visualized by using a Leica DMRB microscope (Leica Microsystems, Rueil-Malmaison, France) equipped with an Olympus DP50 digital camera (Olympus France, Rungis, France). Images were taken (5X for single labelling and 20X for double labelling) standardizing all lighting parameters on the microscope and the camera software (Viewfinder Lite, Olympus) to ensure consistent stable lighting throughout the image capture. Quantifications were performed with Image J software (W. S. Rasband, US National Institutes of Health, Bethesda, Maryland). For single labelling, immunoreactive (IR) neurons within the SCN were quantified separately in dorsal and ventral regions. For double labelling, the number of calbindin neurons co-localizing with c-FOS was counted by an observer blind to the experiments and only those calbindin-positive cells that presented a clear double staining of c-FOS (black nucleus) and calbindin (light brown soma) were included as co-localizing neurons.

2.6. Serotonin and adenosine content in SCN and midbrain of sleep deprived and caffeine treated animals

To conduct this experiment, 24 animals were maintained in LD 12:12 for two weeks and transferred to DD. On the first day of DD, 12 animals were transferred to new cages (without running wheel) at CT12 and 6 out of these 12 animals were sleep deprived by gentle handling (Hubbard et al., 2015) between CT12 and CT16. At the same time, the remaining 12 animals were selected for caffeine induced arousal or VEH treatment. Treatments were administered on the first day of DD. Each animal received an i.p. injection of CAF or VEH at CT14. All animals were killed at CT17 by decapitation. The brains were rapidly removed. Thereafter, SCN region and midbrain were microdissected visually and stored in -80°C.

SCN, midbrain and plasma preparation for LC-MS/MS analysis

Microdissections of SCN and midbrain raphe were homogenized in Ultra pure water and centrifuged at 15,000g. Plasma was centrifuged at 18,000g. Ten pmoles of D3-caffeine, C5-adenosine, and D4-serotonin internal standards were added to each sample prior to the

AccQTag treatment. Only serotonin (5-hydroxy-tryptamine, 5-HT) and D4-serotonin were derivatized on amine functions. The AccQTag derivation protocol was performed according to the manufacturer recommendations (Watters, Guyancourt, France). Concentrations of total soluble protein in the extracts were determined using Bradford method (Protein Assay, Bio-rad, Marnes-la-Coquette, France)

LC-MS/MS instrumentation and analytical conditions

LC-analyses were performed to quantify the presence of caffeine, adenosine and serotonin. Analyses were done on a Dionex Ultimate 3000 HPLC system (Thermo Electron, Courtaboeuf, France) coupled with a triple quadrupole Endura (Thermo Electron). The system was controlled by Xcalibur v. 2.0 software (Thermo Electron). Extracted derived samples were resuspended in 100 µl of AccQTag derivatization kit/formic acid 0.1% and 10µl of the solution was loaded into an Accucore RP-MS column (ref 17626-102130; 100 x 2.1 mm 2.6 µm, Thermo Electron) heated at 35°C. All molecules and their corresponding deuterated compounds were eluted by applying a gradient of buffers A/B. Buffer A corresponded to H_2O 99.9% / formic acid 0.1% (v/v), whereas buffer B was ACN 99.9 %/ formic acid 0.1% (v/v). A linear gradient of 20%-85% of solvent B at 400µL/min during 2.5 min was applied followed by a washing step (0.5 min at 85% of solvent B) and an equilibration step (1 min of 20% of buffer B).

Qualitative analysis and quantification was performed in the selected reaction monitoring mode (SRM). For ionization, 3500V of liquid junction voltage and 292°C capillary temperature was applied. The selectivity for both Q1 and Q3 was set to 0.7 Da (FWHM). The collision gas pressure of Q2 was set at 2 mTorr of argon. For all molecules, the selection of the monitored transitions and the optimization of the collision energy were first determined. The transitions and the corresponding collision energies (CE) used for SRM were the following: m/z 195.2 → m/z 138.1 (CE = 28 eV) for caffeine, m/z 198.2 → m/z 141.2 (CE = 29 eV) for D3-caffeine, m/z 168.2→ m/z 136.1 (CE = 23 eV) for adenosine, m/z 173.2→ m/z 136.1 for C5-adenosin, m/z 347.2 → m/z 171.1 (CE = 38 eV) for 5-HT, m/z 351.2 → m/z 171.1 (CE = 38 eV) for D4-serotonin. Identification was based on precursor ions, daughter ions and retention times. Quantification was done using the ratio of daughter ions response areas of the internal standards. Data were normalized to ml of plasma or to g of proteins.

2.7. Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed by SigmaPlot (version 12, SPSS Inc, Chicago, IL, USA). Significance was defined at $p < 0.05$.

Two-way analysis of variance (ANOVA) was performed to assess the effect of *Treatment* (sleep deprivation versus control; CAF versus VEH) and *Lighting condition* (Dark versus Light pulse) and the *Interaction* between these factors. To compare the 5-HT, adenosine and caffeine contents in SCN, midbrain and plasma after sleep deprivation or caffeine treatment unpaired Student's *t* test were used. When appropriate, post-hoc analysis was performed using Fisher, LSD method.

3. Results

3.1. Early night sleep deprivation induces phase delay shifts and sleep deprivation in early and mid-night potentiates light-induced phase shifts

To evaluate the possible phase-shift of the locomotor activity rhythm in sleep deprived animals, the offsets of the diurnal pattern of locomotor activity were analysed by two-way ANOVA analysis using the factors *Treatment* (sleep deprivation or control) and the *Lighting condition* (dark and light pulse).

Regarding the early-night (CT12-16) sleep deprivation, the behavioural shifts were significantly modified by *Treatment* ($F_{1,20}$=12.79; p=0.002) and *Lighting condition* ($F_{1,20}$ = 24.08; p<0.001), while *Interaction* was not significant ($F_{1,20}$=0.19; p<0.66), indicating that the effect of *Treatment* was independent of the *Lighting condition* (**Fig 1 A − E**). Post-hoc analysis revealed that sleep deprivation in the absence of a light pulse induced a significant phase-delay compared to non-sleep deprived controls (p=0.01) and it significantly enhanced light-induced phase-delays in the early night compared to light-exposed non-sleep deprived control animals (p=0.03). Furthermore, phase-delays in sleep deprived group with light pulse was significantly higher compare to sleep deprived group in dark (p=0.004) and phase-delays in light-exposed control group was significantly higher compare to dark control group (p=0.001).

With respect to sleep deprivation during late-night (CT16-20), phase shifts were modified by *Treatment* ($F_{1,20}$=6.24; p=0.02) and *Lighting condition* ($F_{1,20}$=15.31; p<0.001). The non-significant *Interaction* ($F_{1,20}$=0.18; p=0.67) effect indicates that the effect of treatment was independent of lighting conditions (**Fig 1 F − J**). Post-hoc analysis revealed that late-night sleep deprivation was unable to induce phase-advances significantly larger than those of the dark control animals (p=0.15). By contrast, late-night sleep deprivation significantly potentiated light-induced phase-advances compared to light-exposed and non-sleep deprived controls (p=0.05). Furthermore, phase-advances in sleep deprived group with light pulse was

significantly higher compare to sleep deprived group in dark (p=0.006) and phase-advances in light-exposed control group was significantly higher compare to dark control group (p=0.02).
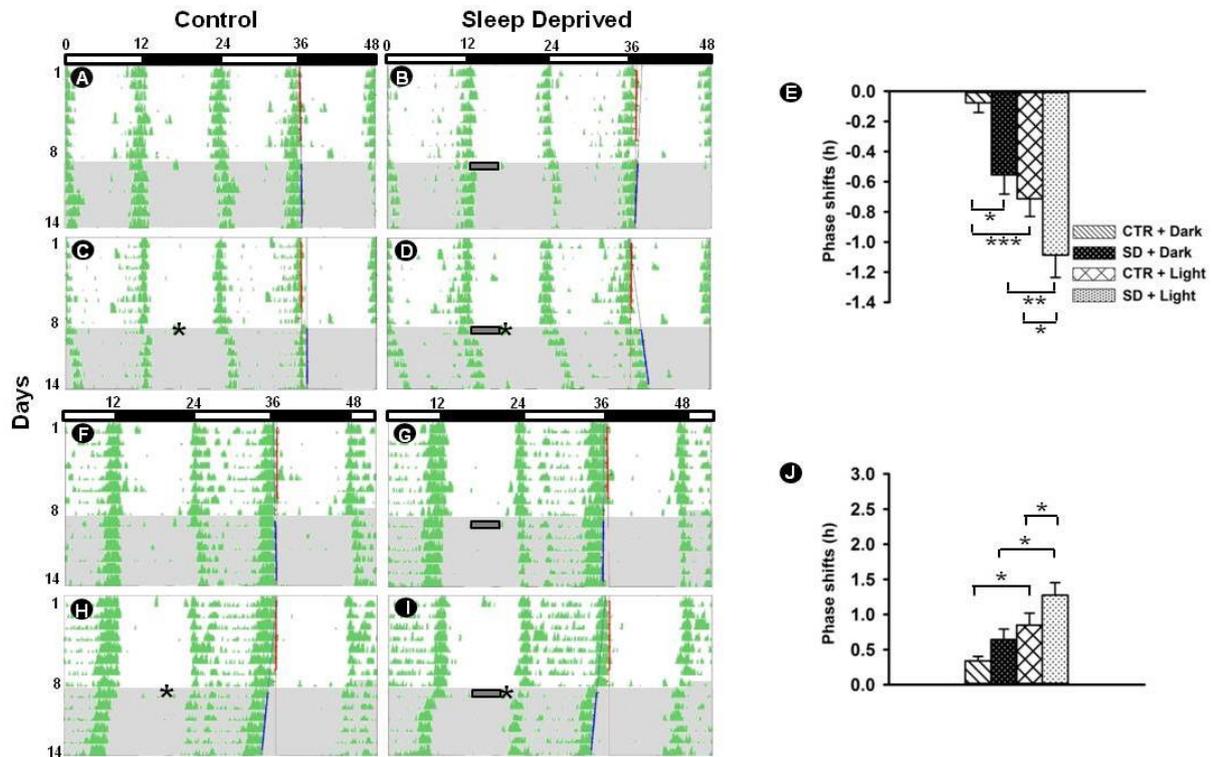

Figure 1 Representative double-plotted actograms of wheel-running activity of eight *Arvicanthis* during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to sleep deprivation, light pulses and the combined effects of sleep deprivation and a light pulse. On the first day of DD, in early night (A-D) and late night (F-I) animals were sleep-deprived or left undisturbed as control, in darkness (upper panels) or followed by light pulse at CT16 and CT20, respectively (lower panels). (E) Mean phase delays (negative value) and (J) phase advances (positive) in response to sleep deprivation, light and the combination of both. An asterisk indicates a statistically significant difference (p < 0.05). Shaded area: DD, rectangular gray bars: time line of sleep deprivation, Stars: light pulse (200 lx for 30 min.), SD = sleep deprivation, CTR = control.

3.2. Daytime caffeine treatment does not induce a phase-shift or influence light induced phase-shifts

Caffeine treatment (30 mg/kg) in the early subjective morning (CT0) and mid-subjective day (CT6) did not induce a phase-shift in the locomotor activity rhythm, or significantly change light-induced phase shifts. ANOVA revealed that caffeine treatment in the early subjective day did not change the effect of *Lighting condition* ($F_{1,20}=7.4$; p=0.01) (**Fig 2 A-E**). Post-hoc analysis indicated that phase-shifts were significantly larger after VEH + Light than after VEH + Dark (p=0.03). After caffeine treatment during mid-subjective day we did not observe any significant effects of *Treatment* ($F_{1,20}=0.47$; p=0.49), *Lighting condition* ($F_{1,20}=0.39$; p=0.53) or *Interaction* ($F_{1,20} = 0.1$; p = 0.74) (**Fig 2 F-J**).
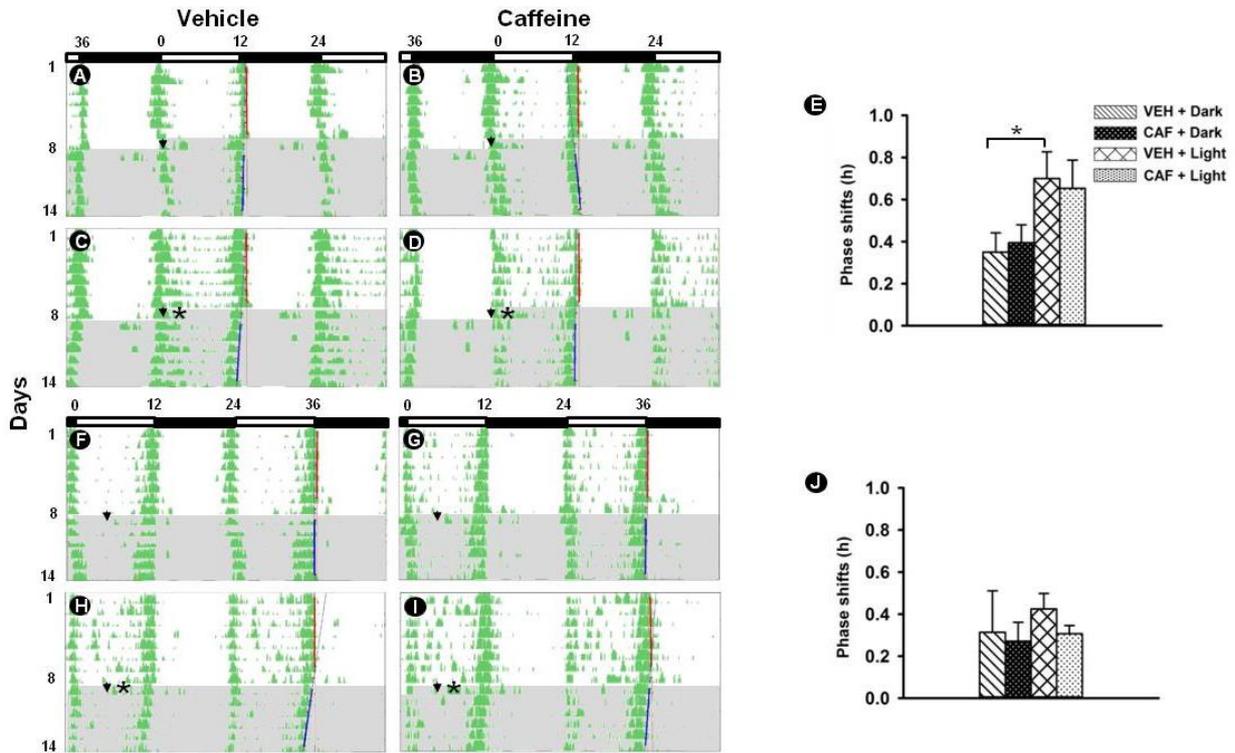
Figure 2 Representative double-plotted actograms of wheel-running activity of eight *Arvicanthis* during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to caffeine treatment, light pulses and the combined effects of caffeine and light pulse. On the first day of DD, in morning at CT0 (A-D) and mid-day at CT6 (F-I) animals were administered caffeine and vehicle in dark (upper panels) or followed by light pulse at CT2 and CT8, respectively (lower panels). Mean phase advances (positive) to caffeine treatment, light pulses and combination of both in the morning (E) and mid-day (J). An asterisk indicates a statistically significant difference ($p < 0.05$). Shaded area: DD, arrows: time of injection, Stars: light pulse (200 lx for 30 min.), CAF = caffeine, VEH = vehicle.

Caffeine treatment in early and mid-night potentiates light-induced phase-shifts

Caffeine treatment in early subjective night (CT14) and mid-subjective night (CT18) potentiated light-induced phase-delays and advances, respectively. ANOVA revealed that both caffeine treatment in the early night (CAF or VEH; $F_{1,20} = 6.4$; p=0.02) and light ($F_{1,20}=40$; p<0.001) induced a significant phase-delay. The non-significant Interaction effect ($F_{1,20}=1.18$; p=0.29) indicates that the effect of caffeine was independent of the lighting condition (Fig 3 A – E). CAF + Light treatment induced a phase-delay that was significantly greater than those after VEH + Light (p=0.01) and CAF + Dark (p<0.001). Phase-delays induced by VEH + Light treatment was significantly higher compare to VEH + Dark (p=0.001).

Similar to early night, caffeine (CAF or VEH; $F_{1,20} = 5.4$; p = 0.03) and light ($F_{1,20} = 40.9$; p < 0.001) treatment during mid-night induced significant phase-advance. The non-significant Interaction effect ($F_{1,20} = 1.18$; p = 0.29) indicates that also during midnight the effect of

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caffeine treatment was independent of the lighting condition (Fig 3 F – J). CAF + Light treatment induced phase-advances significantly greater than those after VEH + Light (p = 0.02) and CAF + Dark (p < 0.001). Phase-advances induced by VEH + Light treatment was significantly higher compare to VEH + Dark (p=0.001).
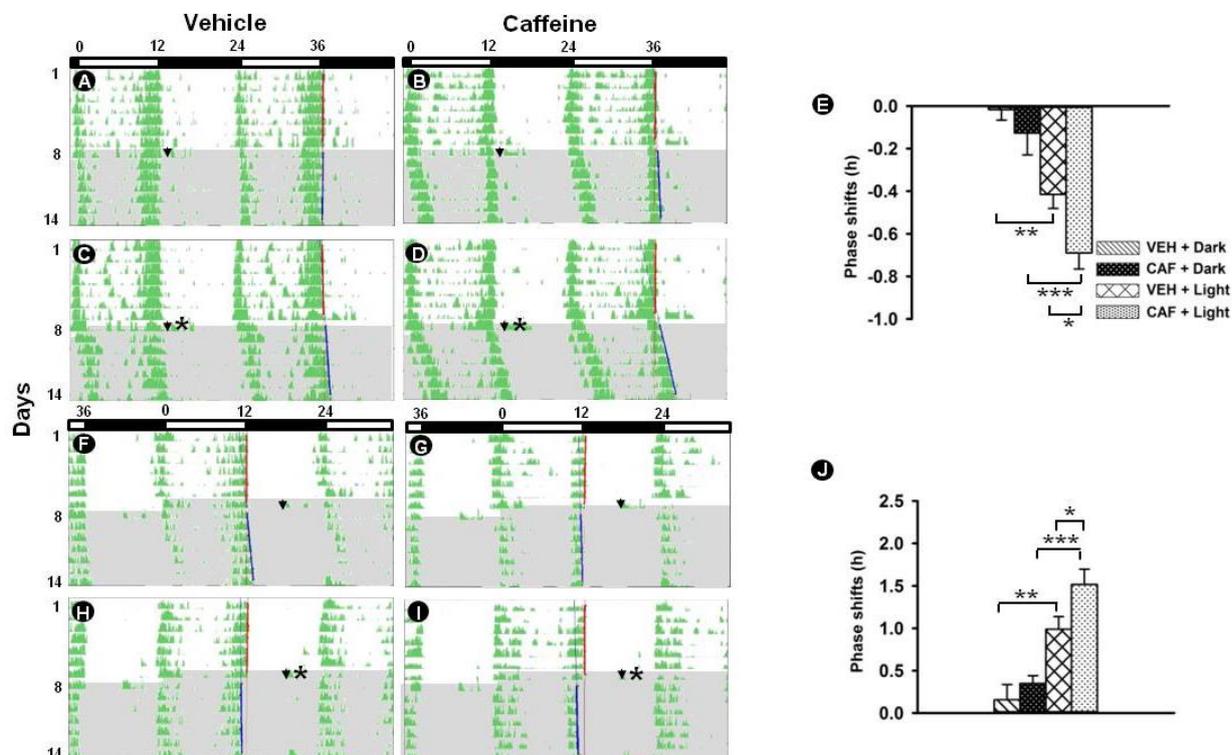

Figure 3 Representative double-plotted actograms of wheel-running activity of eight *Arvicanthis* during 1 week under a light-dark cycle and 1 week in constant darkness (DD), displaying phase-shifts to caffeine treatment, light pulses and the combined effects of caffeine and a light pulse. On the first day of DD, in early night at CT14 (A-D) and midnight CT18 (F-I) animals were administered with caffeine and vehicle in dark (upper panels) or followed by light pulse at CT16 and CT20, respectively (lower panel). (E) Mean phase delays (negative value) and (J) advances (positive) to caffeine treatment, light pulses and combination of both. An asterisk indicates a statistically significant interaction (p < 0.05). Shaded area: DD, arrows: time of injection, Stars: light pulse (200 lx for 30 min.), CAF = caffeine, VEH = vehicle.

Early night sleep deprivation potentiates light-induced c-FOS expression in ventral SCN

Light pulses at CT16 and CT20 increased c-FOS expression in the dorsal as well as ventral SCN, independent of treatment (sleep deprivation or control) (Fig 4 A - D; light effect at CT16; dorsal SCN: $F_{1,16} = 24.48$, p < 0.001; ventral SCN: $F_{1,16} = 210.5$, p < 0.001; light effect at CT20, dorsal SCN : $F_{1,16} = 57.57$, p < 0.001; ventral SCN: $F_{1,16} = 173.99$, p < 0.001). ANOVA detected a trend of effect of early night SD on c-FOS expression in ventral SCN ($F_{1,16} = 3.38$, p = 0.07). Post hoc analysis indicated that at CT16 the light-induced c-FOS

expression in the ventral SCN was significantly greater in sleep-deprived animals than in non-sleep deprived controls (p = 0.03).
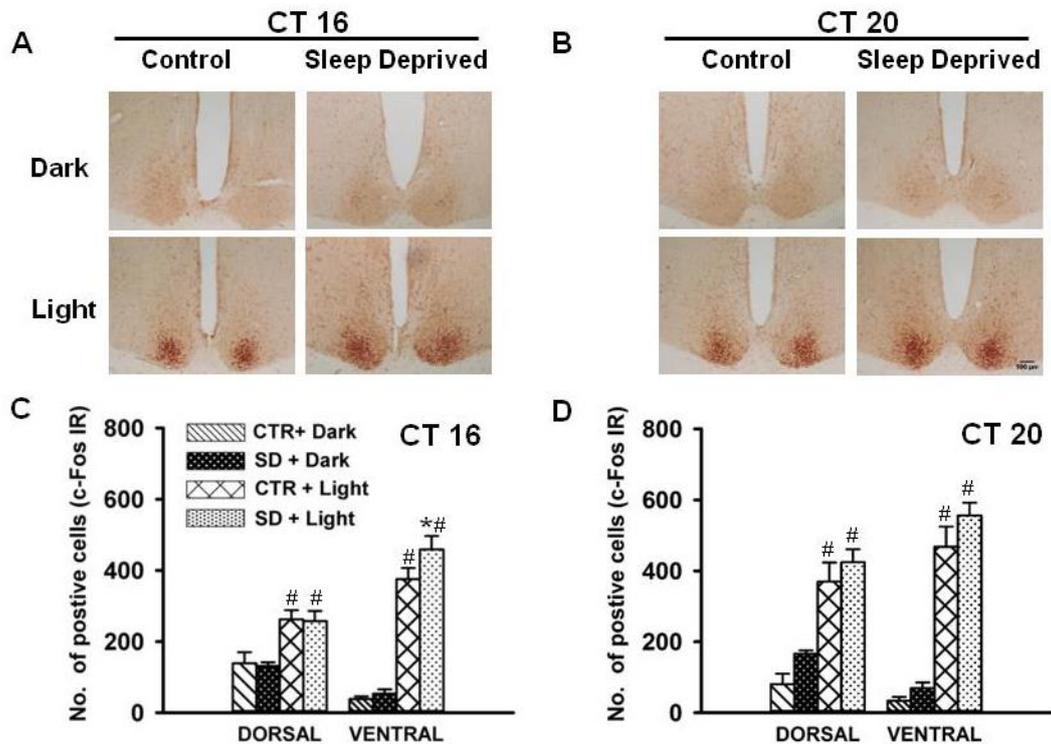

Figure 4 (A, B) Representative photomicrographs of c-FOS in the SCN of early and late-night sleep-deprived and control *Arvicanthis* in darkness or with exposure of light pulse for 30-min at CT16 and CT20, respectively. (C, D) c-FOS mean responses of SCN of early and late-night sleep derived and control *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. * p < 0.05 between sleep-deprived and control groups; # p < 0.001 between light and dark conditions. Scale bar = 100 μm. SD = sleep deprivation, CTR = control.

Caffeine treatment induces c-Fos expression in the dorsal SCN and potentiates light-induced c-Fos expression in ventral SCN

We also examined the effect of caffeine-induced arousal on c-FOS expression in the master clock. During the early subjective night, c-FOS expression in the dorsal SCN was only altered by Lighting condition ($F_{1,20}$ = 7.9; p = 0.01). Post-hoc analysis indicated that number of c-FOS positive nuclei in VEH + Light-treated group were significantly higher compared to dark controls (p = 0.008). In the ventral SCN c-FOS expression was altered by both Treatment (CAF or VEH; $F_{1,20}$ = 5.1; p = 0.02) and Lighting condition ($F_{1,20}$ = 52.8; p = 0.02). Furthermore, post-hoc analysis indicated a significant increase in the number of c-FOS-positive nuclei in the ventral SCN of the CAF + Light group compared to the VEH + Light (p = 0.006) and CAF + Dark (p < 0.001) groups. The number of c-FOS positive nuclei in VEH +

Light treatment group was also significantly higher than in the dark controls (p = 0.001) (Fig 5 A and C).

During mid-subjective night, c-FOS expression in the dorsal SCN was altered by Treatment ($F_{1,20}$ = 6.5; p = 0.01) and Lighting condition ($F_{1,20}$ = 28.5; p < 0.01). Post-hoc analysis indicated a significant increase in the number of c-FOS positive nuclei in the dorsal SCN of CAF + Dark group compare to VEH + Dark (p = 0.01). The number of c-FOS positive nuclei in VEH + Light treatment group was also significantly higher compared to dark controls (p < 0.001). Moreover, in the ventral SCN c-FOS expression was also altered by Treatment ($F_{1,20}$ = 6.8; p = 0.02) and Lighting condition ($F_{1,20}$ = 167.3; p < 0.001). A significant increase in the number of Fos-positive nuclei in the ventral SCN of CAF + Light group was detected as compared to VEH + Light (p = 0.01) and CAF + Dark (p < 0.001) group. The number of c-FOS positive nuclei in VEH + Light group was also significantly higher compared to dark controls (p < 0.001) (Fig 5 B and D).
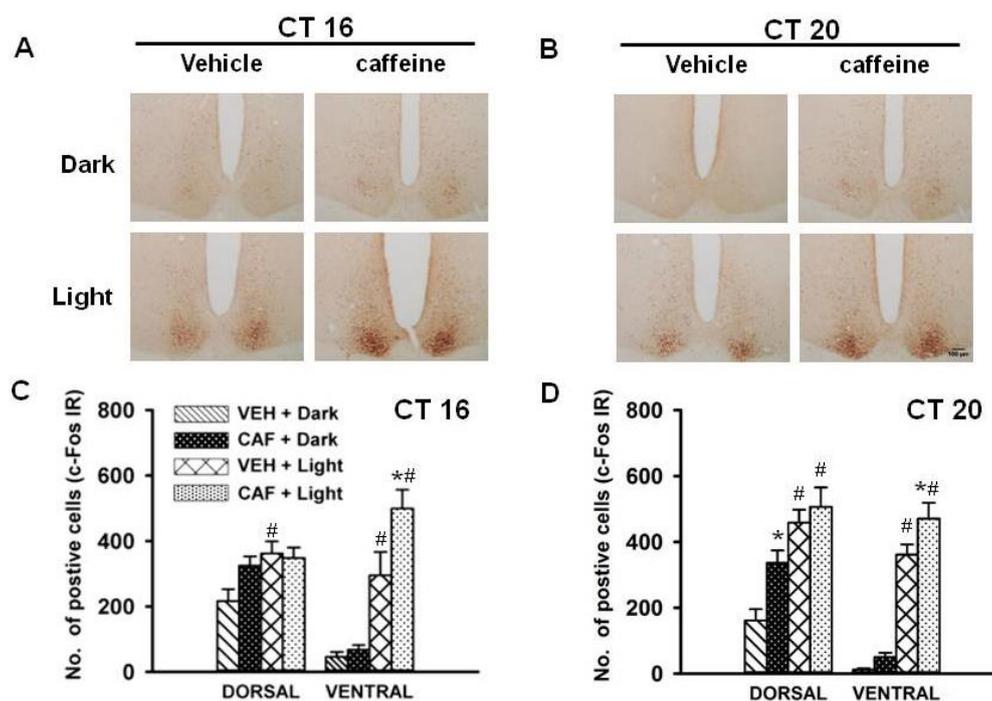
Figure 5 (A, B) Representative photomicrographs of c-FOS in the SCN of early and midnight vehicle- and caffeine-treated *Arvicanthis* in darkness or with exposure of light pulse for 30-minutes at CT16 and CT20, respectively. (C, D) c-FOS mean responses of SCN of early and midnight vehicle and caffeine treated *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. * p < 0.05 between caffeine- and vehicle-injected groups; # p < 0.001 between light and dark conditions. Scale bar = 100 μm. CAF = caffeine, VEH = vehicle.

Sleep deprivation and caffeine treatment alone or with light pulses activate calbindin containing cells in the SCN

To phenotype the activated cells in the SCN by arousal and/or light pulse, we performed double immunolabelling for calbindin and c-FOS. In the dorso-ventral SCN, we calculated the percentage of calbindin cells that showed c-FOS immunoreactivity (IR). Both during early night and mid-night sleep deprivation, ANOVA revealed that the percentage of calbindin cells co-expressing c-FOS IR were modified by Treatment (early night: $F_{1,16} = 37.3$; p < 0.001; mid-night: $F_{1,16} = 153.5$; p < 0.001) and Lighting condition (early night: $F_{1,16} = 46.6$; p < 0.001; mid-night: $F_{1,16} = 420.8$; p < 0.001) (Fig 6 A-C). Post-hoc analysis showed that both in early and midnight, the percentages of calbindin cells showing c-FOS IR were significantly higher in SD + Dark group compared to CTR + Dark (p < 0.001). Moreover, these percentages were also higher in SD + Light compared to CTR + Light (early night: p=0.007; mid-night: p = 0.002) and SD + Dark (early night: p=0.002; mid-night: p < 0.001). In both conditions, CTR + Light group showed a higher percentage of expression than CTR + Dark (p < 0.001).

Figure 6 (A) Representative photomicrographs of the SCN of early night sleep-deprived and control *Arvicanthis* in darkness or with exposure of light pulse for 30-min at CT16, showing c-FOS (dark black nucleus) and Calbindin (light brown soma). Black arrows, single c-FOS immunoreactive (IR) neurons; yellow arrows, single calbindin IR neurons, blue arrows, neurons positive for both c-FOS and calbindin IR. Mean percentage of calbindin neurons with c-FOS IR in the SCN of (B) early night and (C) late-night sleep-deprived and control *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. $ p < 0.001 between sleep deprived and control; # p < 0.001 between light and dark conditions. Scale bar = 10 μm. SD = sleep deprivation, CTR = control.

Similar to sleep deprivation, in both early night and mid-night, ANOVA revealed that the percentages of the calbindin cells showing c-FOS IR were different after caffeine treatment

(early night: $F_{1,20} = 27.3$; $p < 0.001$; mid-night: $F_{1,20} = 134.4$; $p < 0.001$) and light exposure (early night: $F_{1,20} = 12.18$; $p = 0.002$; mid-night: $F_{1,20} = 45.8$; $p < 0.001$) (**Fig 7 A-C**). Post-hoc analysis showed that both in early and mid-night the percentages of calbindin cells co-expressing c-FOS were significantly higher in CAF + Dark group compared to VEH + Dark ($p < 0.001$). Moreover, these percentages were also higher in CAF + Light compare to VEH + Light (early night: $p = 0.007$; mid-night: $p < 0.001$) and CAF + Dark (early night: $p=0.005$; mid-night: $p < 0.001$). In both conditions, VEH + Light showed a higher expression percentage than that of VEH + Dark (early night: $p = 0.005$; mid night: $p < 0.001$). Remarkably the mean effect of caffeine (CAF + Dark versus VEH + Dark) appeared even stronger than that of light (VEH + Light versus VEH + Dark), although this difference (CAF + Dark versus VEH + Light) did not reach significance.
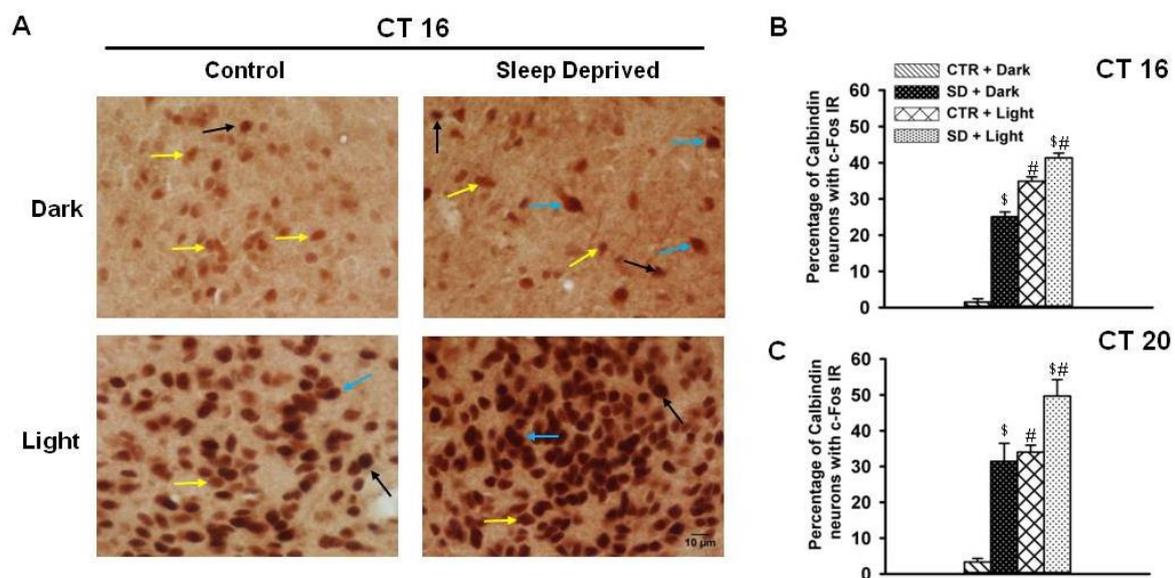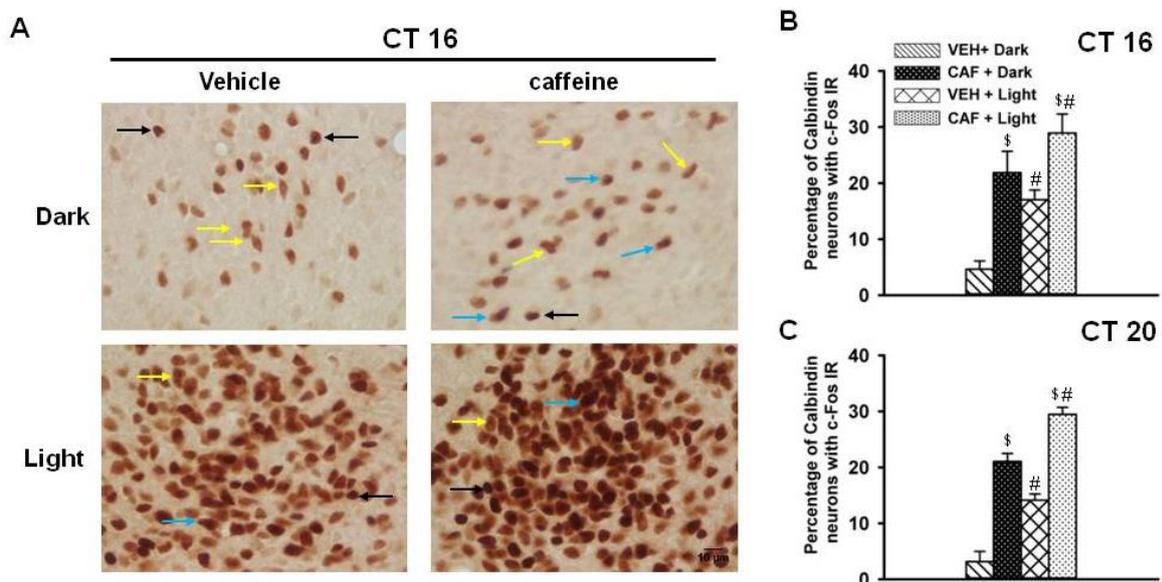

Figure 7 (A) Representative photomicrographs of the SCN of early night vehicle and caffeine treated *Arvicanthis* in darkness or with exposure of light pulse for 30-minutes at CT16, showing c-FOS (dark black nucleus) and Calbindin (light brown soma). Black arrows, single c-FOS immunoreactive (IR) neurons; yellow arrows, single calbindin IR neurons, blue arrows, neurons positive for both c-FOS and calbindin IR. Mean percentage of calbindin neurons with c-FOS IR in the SCN of (B) early night and (C) midnight vehicle and caffeine treated *Arvicanthis* in darkness or with exposure of light pulse at CT16 and CT20, respectively. $ $p < 0.001$ between caffeine and vehicle injected group; # $p < 0.001$ between light and dark conditions. Scale bar ＝ 10 μm. CAF ＝ caffeine, VEH ＝ vehicle.

5-HT and adenosine content in the SCN and midbrain after early night sleep deprivation or caffeine treatment

The levels of 5-HT and adenosine were assessed in SCN and midbrain after sleep deprivation and caffeine treatment during early subjective night (CT 14) (Table 1).

Table 1. 5-HT and adenosine content in the SCN and midbrain after early subjective night of sleep deprivation or caffeine treatment.

	CTR	SD	VEH	CAF
SCN-5-HT (nmol/g of SCN)	40.17±2.66	40.20±3.52	37.57±2.49	44.43±4.75
Midbrain-5HT (nmol/g of midbrain)	7.07±0.69	6.59±0.38	6.05±0.64	8.64±0.59*
SCN-adenosine (µmol/g of SCN)	7.69±1.14	9.71±1.84	7.18±1.2	7.3±1.4
Midbrain-adenosine (µmol/g of midbrain)	22.48±1.62	23.05±1.5	23.08±1.6	22.39±1.5

Data are presented as mean ± SEM (n = 6/group) and were analysed by Student's *t*-test (unpaired, two-tailed); * $p < 0.01$

The levels of 5-HT were not different in SCN and midbrain after sleep deprivation (Fig 8 A). Caffeine treatment enhanced midbrain levels of 5-HT significantly ($t_{12} = 3.22$, $p_{two\text{-}tailed} = 0.007$), though SCN levels of 5-HT remained unchanged after caffeine injection (Fig 8 B). The level of adenosine in SCN and midbrain did not change after sleep deprivation or caffeine treatment (Fig 8 C-D).
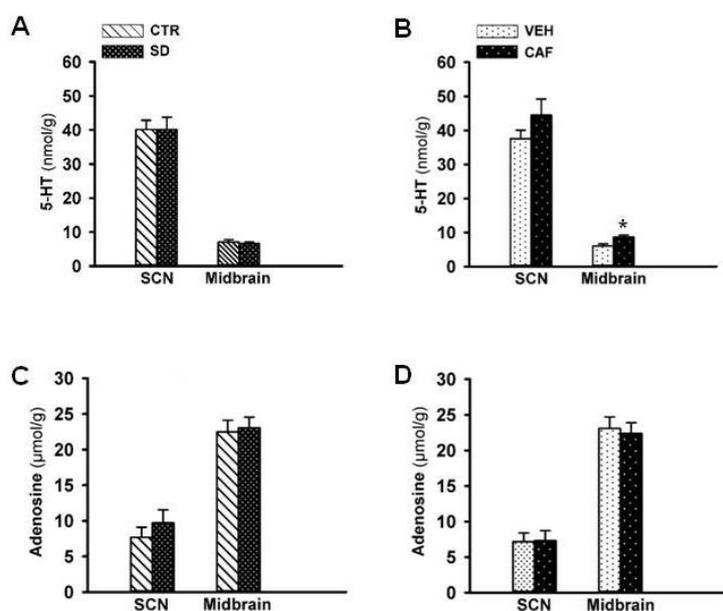

Figure 8 SCN and midbrain contents of (A,B) serotonin and (C,D) adenosine after early night sleep deprivation (CT12-16) and vehicle or caffeine treatments (CT14) in *Arvicanthis*. *p < 0.01 between caffeine and vehicle injected group; SD = sleep deprivation, CTR = control, CAF = caffeine, VEH = vehicle.

Caffeine content in SCN, midbrain and plasma 3 h after caffeine injection in early night

The levels of caffeine in the SCN, midbrain and plasma were quantified 3 h after caffeine or vehicle injection in the early night (Table 2). In addition to plasma, caffeine levels in the SCN and midbrain were markedly elevated in caffeine-treated animals ($p < 0.002$, t-test), indicating that caffeine can directly target these brain areas.

Table 2. Caffeine contents in the SCN, midbrain and plasma 3 h after intraperitoneal injection of vehicle and caffeine in early night.

Treatments	Caffeine in SCN (mmol/g of SCN)	Caffeine in midbrain (mmol/g of midbrain)	Caffeine in plasma (mmol/ml of plasma)
VEH	26.50±14.02	18.93±9.88	0.028±0.02
CAF	2691.54 ±722.35[*]	3180.69±212.37[*]	402.43±88.42[*]

Data are presented as mean ± SEM (n = 6/group) and were analysed by Student's *t*-test (unpaired, two-tailed); * $p < 0.002$

4. Discussion

It is widely acknowledged that behavioural arousal in the usual rest period has the ability to reset the master circadian clock (Webb et al., 2014). For instance, 3 h of sleep deprivation in the middle of the rest period induced phase-advances in Syrian hamsters (Antle and Mistlberger, 2000). Moreover, sleep deprivation and caffeine-induced arousal influence photic resetting of the SCN clock by attenuating light-induced phase-shifts in nocturnal mice and hamsters (Mistlberger et al., 1997; Challet et al., 2001; Vivanco et al., 2013; van Diepen et al., 2014). Most of the experiments supporting these statements have been carried out in laboratory rodents that are essentially night-active. It remains unresolved, however, whether these non-photic stimuli influence the SCN clock and its photic resetting of diurnal species in a similar or different manner. Using sleep deprivation and caffeine treatment as involuntary behavioural arousal, we show here that sleep deprivation in the early night induces phase-delays, whereas both nightly sleep deprivation and caffeine treatment enhance light-induced phase-shifts of locomotor activity rhythm in the diurnal Sudanian grass rat (*Arvicanthis ansorgei*). These behavioural changes are concomitant at the cellular level with potentiation of light-induced c-FOS expression in SCN cells, in particular those containing calbindin. Noteworthy, these findings are conspicuously opposite to the results obtained earlier in nocturnal rodents, i.e., sleep deprivation and caffeine increase instead of decrease light-induced phase-shifts.

Sleep deprivation, but not caffeine, in the early night induces phase-delays in diurnal grass rats

It has been demonstrated that an animal's state of arousal, associated or not with increased locomotor activity, can modulate firing rate of SCN cells, alter the period and shift the phase of circadian rhythms in the nocturnal rodents (Yamada et al., 1986; Reebs and Mrosovsky, 1989a; Antle and Mistlberger, 2000). *In vivo* recordings of extracellular firing in the SCN of freely moving nocturnal rats and hamsters show that locomotor activity suppresses SCN firing rate, with larger effects during subjective day (Meijer et al., 1997; Yamazaki et al., 1998; Schaap and Meijer, 2001). The phase-response curves to arousing stimuli show that both magnitude and direction of the shifts are gated by circadian phase, with maximal phase-advances during the middle of the rest period and minimal phase-delays during the latter half of the usual wake phase (Hughes and Piggins, 2012; Webb et al., 2014). Experiments aimed at mimicking sleep deprivation by intracranial or i.p. injection of an adenosine A1 agonist in the mid-sleep period show dose-dependent shifts similar to those induced by sleep deprivation itself. In addition, the adenosine receptor antagonist caffeine attenuates the phase-shifts produced by sleep deprivation in nocturnal rodents (Antle et al., 2001).

In the present study, we found that caffeine alone does not induce significant behavioural shifts in diurnal grass rats, in keeping with an earlier study in nocturnal rodents (Vivanco et al., 2013). In humans, however, caffeine intake in the early night produces a phase-delay of the circadian melatonin phase (Burke et al., 2015). In parallel, we showed that sleep deprivation in the early subjective night, without any notable hyperactivity, induces a phase-delay of the behavioural rhythm in diurnal grass rats. These findings are, to some extent, reminiscent of previous studies in humans that showed that physical activity in early evening and mid-night induces phase-advances and delays, respectively (Buxton et al., 1997; Buxton et al., 2003; Mistlberger and Skene, 2005). On the other hand, sleep deprivation later in the subjective night (CT 16-20) does not produce significant shifts in the diurnal grass rats compared to undisturbed controls. Together, these data indicate that the overall shape and timing of phase-responses to sleep deprivation in nocturnal and diurnal species depend on the phase of the rest-activity cycle and are phased at opposite circadian times in both species.

Sleep deprivation and caffeine treatment potentiate light-induced shifts in diurnal grass rats

Responses of the mammalian circadian system to both photic and non-photic stimuli are mediated by effects on the central SCN oscillator. Unlike photic and arousal independent stimuli, however, in diurnal species non-photic stimuli dependent on arousal, provide

synchronizing feedback signals to the SCN oscillator in circadian anti-phase and show opposite modulatory effects on light-induced phase-shifts as compared to nocturnal animals (Challet, 2007; Cuesta et al., 2008). Light exposure at night increases SCN firing rate and opposes the motor activity-induced suppression of SCN firing rate in nocturnal rats (Schaap and Meijer, 2001). By contrast, sleep deprivation in mice attenuates the sustained response to light on SCN firing rate, while caffeine treatment normalizes the light response again (van Diepen et al., 2014).

In nocturnal species, acute sleep deprivation by procedures not involving increased motor activity (e.g., gentle handling), forced activity, or injections of an adenosine A1 agonist inhibit light-induced phase-shifts (Watanabe et al., 1996; Mistlberger et al., 1997; Challet et al., 2001; Elliott et al., 2001; Sigworth and Rea, 2003). On the other hand, the adenosine antagonist caffeine also blocks light-induced phase-delays in the mice (Vivanco et al., 2013). Note that the dose used in that study (40 mg/kg) was unable to induce a phase shift. In Syrian hamsters, even higher doses of caffeine (75 mg/kg) did not trigger significant phase-shifts, but they did reduce the phase-shifts induced by sleep deprivation (Antle et al., 2001).

Using gentle handling as a procedure for sleep deprivation, a procedure which does not induce significant physical movements, we here show that 4 h of sleep deprivation in the early and mid-subjective night enhances light-induced phase-delays and advances in the diurnal rodent, *Arvicanthis*. Furthermore, our data show similar potentiating effects of caffeine (30 mg/kg) on light-induced phase-shifts. These findings are opposite to the reducing effects found in nocturnal rodents (Vivanco et al., 2013). In humans, however, caffeine intake in the early night does not significantly enhance the light-induced phase-delay of the circadian melatonin phase (Burke et al., 2015). Together, the results suggest that behavioural arousal, induced by sleep deprivation or caffeine treatment, differentially affects photic resetting of the circadian master clock in nocturnal and diurnal rodents and in humans. It is worth mentioning that the modulatory effects of sleep deprivation and caffeine on photic resetting in nocturnal and diurnal rodents are functionally comparable to those observed after serotonergic activation (Morin, 1999; Challet, 2007; Cuesta et al., 2008).

5-HT and adenosine levels in SCN and midbrain after sleep deprivation and caffeine treatment in the diurnal grass rats

The activity of the central serotonergic and adenosinergic systems being closely related with behavioural arousal, they may participate in the non-photic resetting of the SCN by arousing cues. Serotonin (5-HT) concentrations in the SCN follow a daily pattern with higher levels during the active phase in both nocturnal and diurnal rodents (Poncet et al., 1993; Cuesta et

al., 2008). Previous studies in nocturnal animals indicated that sleep deprivation stimulates release of 5-HT and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) in SCN, raphe nucleus and forebrain (Toru et al., 1984; Asikainen et al., 1995; Grossman et al., 2000; Challet et al., 2001). The increased concentration of 5-HT or 5-HIAA in the SCN and other hypothalamic areas during sleep deprivation was also correlated to circadian phase-shifts and/or attenuated light-induced phase-shifts in nocturnal rodents (Grossman et al., 2000; Challet et al., 2001). Furthermore, methamphetamine, a wake-promoting drug, increases the release of serotonin in the SCN and also reduces photic responses of the SCN in nocturnal rats (Moriya et al., 1996; Ono et al., 1996). In the current experiment 5-HT concentrations after 4 h of sleep deprivation in the early subjective night, as measured in tissue blocks, remained unchanged in the SCN and midbrain of diurnal rodents, thus suggesting that arousal feedback would be mediated by other mediators (e.g., adenosinergic cues).

Unlike serotonin, adenosine is widely accepted as an inhibitory neuromodulator within the brain. Extracellular levels of adenosine increase after sleep deprivation in the cortex and basal forebrain and decrease during the sleep recovery period (Huang et al., 2014). In nocturnal rodents the adenosine receptor antagonist caffeine attenuates the phase-advances induced by sleep deprivation and blocks light-induced phase-delays. In addition, microinjection of an adenosine agonist in the SCN of hamsters and rats induces phase-shifts, as does sleep deprivation, and attenuates light-induced phase-shifts (Watanabe et al., 1996; Antle et al., 2001; Elliott et al., 2001). Unexpectedly, similar effects on light-induced phase-shifts were reported after caffeine treatment, i.e., an adenosine antagonist (Vivanco et al., 2013). Some studies reported the presence of adenosine receptors in the SCN (Chen and van den Pol, 1997; Diaz-Munoz et al., 1999; Hallworth et al., 2002), suggesting that caffeine may act directly on SCN cells. Ryanodine receptors (RyRs) are caffeine-sensitive Ca^{2+} release channels (McPherson et al., 1991). Caffeine-mediated increases of Ca^{2+} levels in SCN neurons are dependent on the activation of RyRs (Pfeffer et al., 2009). Ryanodine and caffeine treatment applied in vitro during the subjective day produce phase-advances in the hamster SCN (Diaz-Munoz et al., 1999). These finding indicate that the effects of sleep deprivation and *in vivo* caffeine treatment may have been achieved by activation of the adenosinergic system in the SCN. The present data do not fully support this hypothesis because adenosine levels in SCN and midbrain of grass rats, either sleep-deprived or treated with caffeine, remained essentially unchanged. On the other hand, tissue measurements do not accurately reflect release. Of note, while 5-HT levels remained unchanged in the SCN region, caffeine treatment in early night significantly increased 5-HT concentrations in the midbrain raphe. This confirms the fact that

caffeine promotes serotonergic activation (Antle et al., 2001; Okada et al., 2001; Vivanco et al., 2013). Together these findings suggest that arousal feedback may also be mediated by mediators other than serotonergic and adenosinergic pathways (e.g., orexinergic cues). Further studies using intra-cerebral microdialysis of 5-HT, adenosine and orexin are needed to solve this issue.

Cellular responses of arousal on light sensitivity of SCN

Nocturnal and diurnal species display similar temporal patterns of light-induced c-FOS expression in the SCN (Mahoney et al., 2001). Photic stimulation of c-FOS expression in the SCN has been used by many labs to study the photosensitive phase of the master clock. Previous studies in nocturnal species have shown that arousal changes cellular activity in the SCN and influences its photic sensitivity. Levels of c-FOS expression in the SCN of nocturnal animals decrease when they are aroused during their usual resting period (Mistlberger et al., 1998; Antle and Mistlberger, 2000). Activation of the adenosine A1 receptor attenuates light-induced c-FOS and p-ERK expression in the SCN of nocturnal rodents (Watanabe et al., 1996; Sigworth and Rea, 2003). Interestingly, similar results have been obtained after treatment with other wake-promoting drugs, such as methamphetamine (Moriya et al., 1996; Ono et al., 1996). Our results in the diurnal grass rat show that sleep deprivation in the early subjective night increases light-induced c-FOS induction in the ventral SCN. During mid-subjective night there was a trend for such an enhancement. Caffeine treatment in the mid-subjective night induces c-FOS expression in the dorsal SCN, albeit without inducing a subsequent phase-shift. Furthermore, caffeine treatment in the both early and mid-subjective night potentiated light-induced Fos expression in the ventral SCN. These data show that the feedback effects of arousal on SCN cellular activity and their interaction with the light-induced expression of immediate early genes are opposite in nocturnal and diurnal rodents.

Our results with the double immunolabelling indicate that both sleep deprivation and caffeine treatment activate a large proportion of the calbindin-containing cells in the SCN of diurnal rodents. Earlier studies in nocturnal hamsters have already involved SCN calbindin in the maintenance of circadian rhythms and photic entrainment (Hamada et al., 2003; Kriegsfeld et al., 2008; Stadler et al., 2010). For instance, administration of calbindin-antisense oligo-deoxynucleotides inhibited light-induced phase-shifts and *Per* expression in the SCN during the subjective night and enhanced these parameters during the subjective day (Hamada et al., 2003). Moreover, targeted mutation of the D_{28K} gene lead to a disruption of circadian rhythms and entrainment by overall reduction of light-induced c-FOS expression in the SCN (Kriegsfeld et al., 2008). Our results are the first to suggest an involvement of SCN calbindin-

containing cells in the feedback effect of arousal on the master clock and its light-induced phase shifts also in diurnal species.

Clock resetting by arousal: perspectives in humans

In the current study sleep deprivation by gentle handling in the early night induced phase-delays. This effect is reminiscent of the shifting properties of physical activity after midnight in humans (Buxton et al., 1997). Moreover, our findings in the diurnal rodent Arvicanthis ansorgei also confirm the chronobiotic inefficacy of caffeine treatment alone in constant darkness, while it potentiates the light-induced phase-shifts. This raises the possibility that nocturnal and diurnal species share common afferent pathways conveying arousing cues to the SCN clock, while they might have distinct mechanisms for modulating the clock and its photic resetting. Clearly the present observations on the chronobiotic effects of arousing cues in a diurnal rodent may be of biomedical relevance.

Several studies in humans have already reported significant effects of arousal or exercise on the circadian system. Daily exercise facilitates phase-delays of melatonin secretion (Barger et al., 2004). Van Cauter and colleagues have shown that physical activity in early and mid-night induces respective phase-advances and delays (Van Reeth et al., 1994; Buxton et al., 1997), highlighting clear phase-dependent effects. The combination of exercise and bright light, however, does not result in a significant increase in the shifts compared with bright light alone (Youngstedt et al., 2002). With respect to the chronobiotic effects of sleep deprivation in humans, partial sleep loss combined with bright light reduces light-induced phase-advances (Burgess, 2010), in contrast to the lack of effect of the combined treatment in diurnal grass rats in late night. To our knowledge, the combination of partial sleep deprivation and light in early night has not been performed in humans yet, thus precluding a precise comparison with the early night data in grass rats. Also the effects of caffeine consumption on the human circadian system have been documented. A recent study in blind patients indicates that daily administration of caffeine (150 mg) is not efficient to entrain the circadian clock (St Hilaire and Lockley, 2015). However, caffeine consumption alone can suppress melatonin release and attenuates the normal decrease in body temperature in sleep-deprived subjects (Wright et al., 1997). Furthermore, evening intake of caffeine (3 mg/kg) can produce significant phase-delays of the human SCN clock, while it does not modify significantly the shifting effect of bright light (Burke et al., 2015). Considering our results in the diurnal grass rat, it could be interesting to test whether caffeine consumption in humans may not potentiate the shifting effects of light exposure with lower intensities.

To sum up, the present study in a diurnal rodent revealed that sleep deprivation can phase-delay the master clock and both sleep deprivation and caffeine administration potentiate light-induced phase-shifts. This work reinforces the view that modulation of the SCN clock by arousing cues may be a key function to maintain the respective temporal niche in day-active and night-active species. Moreover, the availability of a diurnal rodent model could be useful not only for comparative studies with nocturnal rodents and reciprocal interactions with human circadian research, but also for biomedical applications.

Acknowledgements

GENERAL DISCUSSION

In order to be able to anticipate cyclic changes in the environment, such as light and dark, circadian clock mechanisms play an important role in the regulation of many behavioral, metabolic and physiological processes. In mammals the timing of energy intake and expenditure varies according to their sleep/wake and fasting/feeding periods. In both diurnal and nocturnal species, the period of sleep and fasting corresponds to a state of low metabolism and catabolism, whereas wakefulness and feeding coincide with a high metabolic, anabolic and thermogenic state (Alberts et al., 2006; Yang et al., 2010). Calorie intake during the active period ensures the uptake and storage of energy substrates such as carbohydrates and lipids necessary to maintain a high metabolic rate, while during the resting period, stored substrates such as glycogen and fat are metabolized to maintain basal energy expenditure (Kumar Jha et al., 2015).

The hypothalamus controls major components of energy homeostasis, including food and water intake, thermoregulation and energy metabolism, as well as sleep and arousal. Lesion and grafting studies identified the suprachiasmatic nuclei (SCN) in the anterior hypothalamus, as the central clock which controls behavioral and physiological day/night rhythms including sleep/wake, fasting/feeding, glucose metabolism and insulin secretion (Weaver, 1998; Dibner et al., 2010). Circadian regulation of metabolic and physiological processes is well documented. For instance, plasma glucose concentration, glucose tolerance and insulin sensitivity show daily rhythms. Evidence from SCN lesions and denervation of autonomic projections has established the role of the SCN, via the autonomous nervous system, in the circadian modulation of glucose homeostasis (Kalsbeek et al., 2014). The SCN receive direct light information from the retina via the retinohypothalamic tract to synchronize the endogenously generated rhythms of behavior, metabolism and physiology with the environmental light/dark cycle (Kumar Jha et al., 2015).

The SCN clock that controls the behavioral and metabolic rhythms also receives feedback signals from these behavioral and metabolic rhythms. This feedback information may influence period and phase of SCN rhythms. In normal conditions this feedback information will enhance the amplitude of the clock rhythms and strengthen its output. However, metaphorically, the hands of the clock can also be twisted by these behavioral and metabolic cues, when they are not in phase with the clock rhythm. In the long run, perturbations and

misalignments of circadian rhythms may affect physiological and metabolic homeostasis. Sleep and arousal are the most conspicuous rhythmic behaviors in mammals and both also provide feedback to the SCN. Indeed, sleep loss not only impairs metabolic homeostasis but it also affects the SCN clock and its ability to entrain to light. Most studies into these functions have been carried out in nocturnal laboratory animals, whereas only sparse information is available from diurnal animals. In this section of the thesis, we will first discuss the role of the SCN and its neuropeptides in the maintenance of glucose homeostasis. The second part of this Discussion will be focused on how behavioral arousal affects the master clock and its photic resetting, as well as its effect on glucose metabolism, in both nocturnal and diurnal species.

1. SCN neuropeptides and glucose homeostasis

The study described in Chapter 2 is an extension of earlier experiments investigating the involvement of SCN neuropeptides in the control of glucose homeostasis. These earlier studies have shown the implication of a number of hypothalamic neuropeptides, such as AVP, VIP and PACAP in the control of glucose metabolism (Kalsbeek et al., 2010c). In this section, we will discuss the role of GRP in the maintenance of glucose homeostasis.

Previous results indicated that a lack of GRP-R signaling affects glucose metabolism (Persson et al., 2000). The experiments described in Chapter 2 revitalized the concept that GRP-mediated signals in the CNS participate in the control of glucose metabolism. Our data show that an increased availability of GRP in the CNS increases plasma glucose concentration through an increase in hepatic glucose production, probably by stimulating pancreatic glucagon release. From the results described in Chapter 2, it is not clear where in the brain GRP acts to cause hyperglycemia. Activation of c-FOS and p-ERK IR has been detected in the SCN and PVN after central treatment with GRP (Kallingal and Mintz, 2014). It is thus tempting to speculate that hyperglycemia induced by ICV GRP could be mediated at least in part by SCN and/or PVN action on the sympathetic input to the liver (Kalsbeek et al., 2004), although it is not known which neurons in the SCN and PVN are activated. However, based on the observations detailed below we hypothesize that the GRP-induced hyperglycemia may involve an activation of PVN neurons by GRP resulting in increased sympathetic inputs to the pancreas and liver, thus leading to subsequent increased glucagon release and EGP.

The SCN-mediated rhythmic activation and inhibition of pre-autonomic neurons in the PVN is an important part of the mechanism that causes the daily variations in plasma glucose concentrations (Kalsbeek et al., 2008a). GRP-containing neurons located in the SCN amongst others project to the PVN, which is an integrator of SCN signals in the control of plasma glucose rhythm (Kalsbeek et al., 1993). Interestingly, the expression of GRP-R and its binding

also exhibits a daily rhythm in the SCN, with a peak in the early night similar to plasma glucose concentrations (Karatsoreos et al., 2006), though it is not clear whether GRP-R shows a rhythmic expression in PVN or not. The daily variation in GRP-R makes it possible that ICV administration of GRP at different times of the day may have differential effects on plasma glucose level. It is expected that GRP infusion might display more robust effects toward the end of the light period and beginning of the activity period. Further experiments aiming at specifically targeting the PVN with GRP or its antagonists at different times of the day may help to understand the mechanisms underlying GRP-induced hyperglycemia in more detail and whether it is involved in the control of the daily rhythm in plasma glucose concentration. Moreover, liver and/or pancreas denervation studies in GRP-treated animals are needed to delineate further the possible neuronal pathways involved.

As aforementioned, the daily rhythm of plasma glucose concentrations is induced by a mechanism in which the time-of-day dependent excitatory and inhibitory signals from the SCN to pre-autonomic neurons in the PVN result in the rhythmic activation of sympathetic input to the liver (Kalsbeek et al., 2010a). Whether or not the SCN neuropeptides are also implicated in the above pathway is not known (**Figure 1**). The excitatory effect of AVP on the glucose-responsive neurons of ventromedial hypothalamic nucleus may link the SCN AVP system to glucose metabolism (Kow and Pfaff, 1986). It has been further shown that administration of micro-dose of AVP in the nucleus of the tractus solitaries of rat results in hyperglycemia (Yarkov et al., 2001). However, infusion of V1a receptor antagonist in the PVN also resulted in mild hyperglycemia; but this might involve increased release of AVP in the circulation (Kalsbeek et al., 2004). More recently, Song et al.(2014) showed that AVP neurons in the supraoptic nuclei act as glucose sensors, as explants of the hypothalamo-neurohypophyseal system released AVP when treated with glucose and insulin. Other studies indicated that central treatment with PACAP-38, VIP and GRP induces hyperglycemia as well as c-FOS expression in the PVN (Yi et al., 2010; Kallingal and Mintz, 2014). Of note, PACAP-38 induced c-FOS expression in PVN neurons that, amongst others, project to the sympathetic preganglionic neurons in the spinal cord. In the PVN, neurons that connect to the sympathetic and parasympathetic branches of the autonomic nervous system are separated (Buijs et al., 2003b). As for now only limited evidence is available to indicate that the VIP-induced hyperglycemia is mediated by activation of sympathetic activity (Nagai, 2004). Hyperglycemia induced by ICV PACAP-38 can be blocked by selective hepatic sympathetic, but not parasympathetic, denervation (Yi et al., 2010). Further experiments are necessary to

pinpoint the exact mechanisms by which these peptidergic signals regulate glucose metabolism.
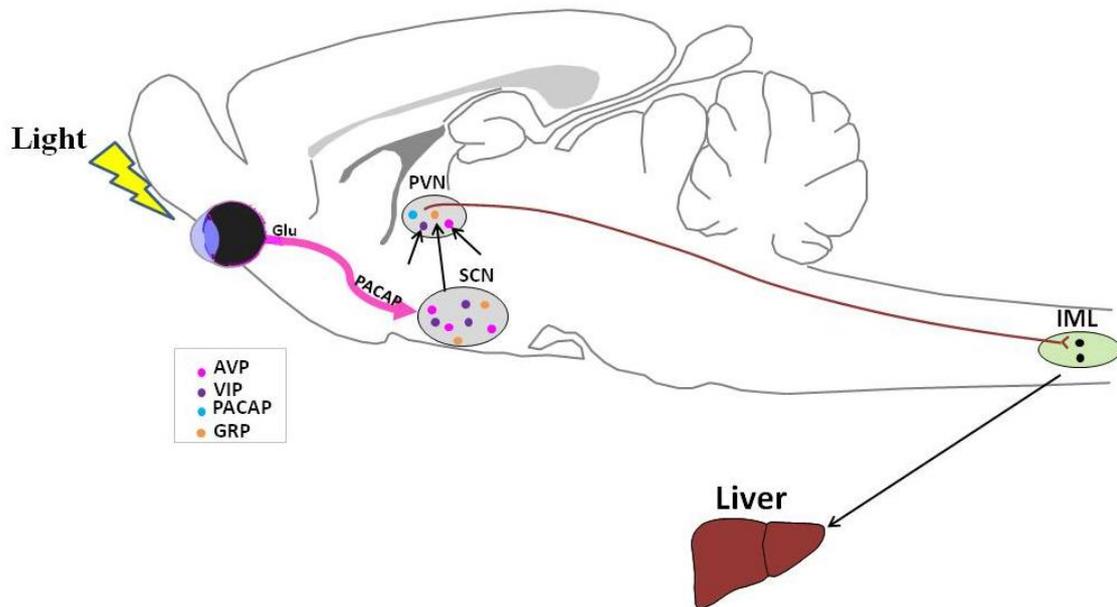

Figure 1. Mid-sagittal view of the rat brain with a schematic representation of proposed AVP, VIP, GRP and PACAP connections involved in the control of glucose metabolism

The neuropeptides project from the SCN and possibly from other hypothalamic areas to the PVN. ICV administration of GRP, VIP and PACAP results in activation of sympathetic inputs to the liver through the intermediolateral column (IML) of the spinal cord stimulating hepatic glucose production.

The daily rhythm of plasma glucose concentration is oppositely phased between nocturnal and diurnal species, having a peak at the beginning of the active period in both species (Kumar Jha et al., 2015). There is no evidence that SCN neuropeptides vary in opposite phase in nocturnal and diurnal species; though the release pattern of most of these SCN neuropeptides in nocturnal rodents is not known in detail and has not been studied in diurnal animals at all. Future experiments aimed to study the daily rhythms of release of these SCN neuropeptides in nocturnal and diurnal rodents in more detail may help to understand how the oppositely phased plasma glucose rhythm in nocturnal and diurnal species is created. As a start, it would be interesting to investigate the effects of GRP, VIP and orexin on glucose metabolism in a diurnal species such as the Sudanian grass rat, *Arvicanthis ansorgei*.

2. Procedures of behavioral arousal

Various methods of sleep deprivation and drug-induced arousal have been employed to study the effect of sleep restriction and arousal on metabolism and circadian rhythms in rats, mice and hamsters. In Chapter 3, we investigated the effect of acute sleep deprivation or caffeine treatment on glucose metabolism in nocturnal rats. In Chapter 4, we studied the effects of

acute sleep deprivation and caffeine-induced arousal on circadian rhythms and light-induced phase-shifts in diurnal grass rats. In both studies, we investigated the impact of 4 h of sleep deprivation during the early and late part of the resting period.

For sleep deprivation, we selected the method of "hand deprivation" or "gentle handling", where we did not allow the animals to fall asleep during either the early or late phase of the resting period. Animals were gently touched, given objects to play or fondled as necessary to keep them awake. Among the several procedures of sleep deprivation described earlier in rodents, including disk-over-water, forced locomotion, rotating drum and some automatic sleep deprivation devices, gentle handling is considered the best way for the short-term sleep deprivation as it helps to reduce the confounding effects of triggered locomotion and increased levels of plasma corticosterone in the animals. Of note, previously gentle handling during 6 h starting at midnight has been proven to be efficient in the diurnal grass rats to induce sleep deprivation and a sleep rebound afterwards (Hubbard et al., 2015). Certain limitations have been also reported from the researchers using "gentle handling" for sleep deprivation which include animal's quick adaptability or unresponsiveness towards the handling and introduction of new playing objects. However, the likelihood of these limitations is minimal for short duration of sleep restriction. In order to mimic the sleep deprivation and study the chronobiotic efficacy of adenosine antagonist, we used caffeine as an arousal-promoting drug in the diurnal grass rat, *Arvicanthis ansorgei*. A previous study in mice suggested that a 20 mg/kg dose of caffeine is unable to affect light-induced phase-shifts and a higher dose of 40 mg/kg blocks the light-induced phase-shifts (Vivanco et al., 2013). Caffeine alone did not induce any phase-shift in mice for doses ranging from 20 to 40 mg/kg. In hamsters, a dose of 75 mg/kg was shown to be inefficient to shift the SCN clock (Antle et al., 2001). Considering these studies, we used 30 mg/kg dose of caffeine in *Arvicanthis ansorgei*. Validation of 4-h sleep deprivation and caffeine-induced arousal and rebound sleep by EEG and EMG recordings is in progress.

3. Sleep deprivation and glucose metabolism

Sleep is considered as a behavioral state of energy conservation and replenishment of energy stores (Roth et al., 2010). The process of sleep and wakefulness is controlled in part by the circadian clocks and in part by homeostatic mechanisms. Various epidemiological and laboratory studies in humans have linked reduced sleep duration, quality and circadian misalignments to abnormal glucose metabolism, increased risk of diabetes and obesity. In Chapter 3, we addressed the issue of how acute sleep deprivation in the early and middle of the rest period affects glucose metabolism in nocturnal rats. Our results demonstrate that four

hours of sleep deprivation in the early or middle of the rest period is sufficient to reduce the acute insulin response to a glucose load and impair glucose tolerance. On the other hand, the basal levels of plasma glucose, insulin and corticosterone remained unchanged after 4 h of sleep deprivation. Throughout the IVGTTs corticosterone concentrations remained unchanged between sleep deprived and control animals. Previous experiments conducted in rats showed that chronic sleep disturbance for 8 days resulted in impaired glucose tolerance caused by a reduced insulin response (Barf et al., 2010). In our study, although the acute insulin response was reduced, the overall insulin response during the IVGTTs in the early and late rest period was not changed in the sleep-deprived group compared to its control group. This disparity in the insulin responses may be due to gradual changes in brain and body after chronic versus acute sleep deprivation.

In parallel to sleep deprivation, we also investigated the effects of caffeine-induced arousal in the rest period on glucose tolerance in rats. Human studies have shown that acute caffeine treatment impairs insulin sensitivity in dose-dependent manner (Beaudoin et al., 2013). On the other hand, chronic treatment improves glucose tolerance in diabetic rats (Urzua et al., 2012). However, the data from our experiments show that acute administration of caffeine in the animals' early or middle rest phase does not affect glucose tolerance (**Figure 2**). Nevertheless, we think these findings are worth mentioning here because they suggest that the effects of sleep deprivation cannot be mimicked with an arousing drug (i.e., the impact of sleep deprivation on glucose tolerance is probably not due to arousing cues).
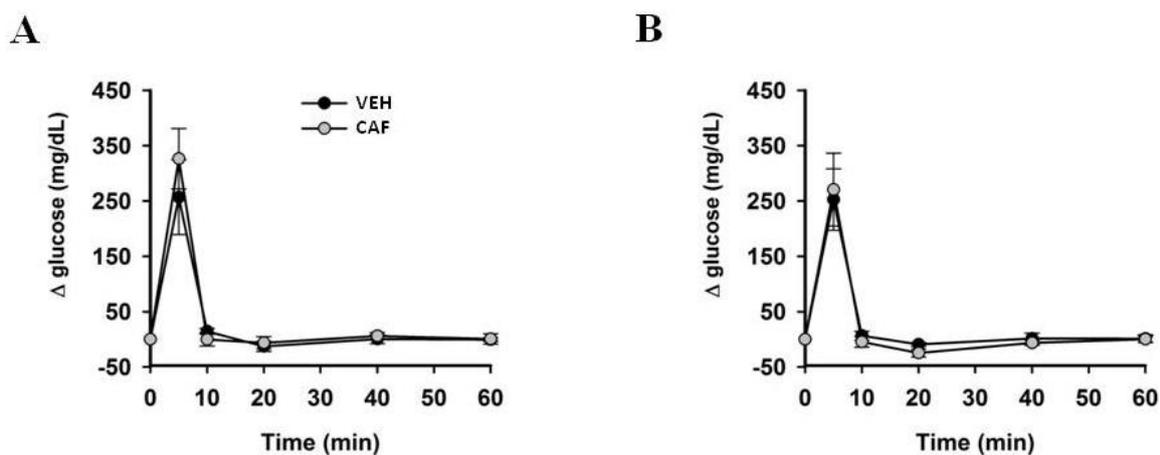

Figure 2. Relative change in plasma glucose concentration in intravenous glucose tolerance tests (IVGTTs) at CT 4 (A) and CT 8 (B) in rats after caffeine treatment (all groups n = 6)

Glucose metabolism is under the control of the circadian clock in the SCN, but is also affected by the clocks in peripheral tissues. Plasma glucose concentrations peak and glucose tolerance

is enhanced at the beginning of the active period (la Fleur et al., 2001a; Kalsbeek et al., 2010a). The peak of glucose concentration at the beginning of the active period is postulated to result from increased hepatic glucose production as well as low insulin secretion. Results from glucose tolerance tests indicate a higher glucose disposal rate at this period, indicating glucose production exceeds glucose uptake in skeletal muscles and adipose tissues (Boden et al., 1996; Kalsbeek et al., 2010a). The circadian system is affected by sleep/wake alterations, including sleep deprivation. The chronic sleep loss may affect glucose homeostasis through different pathways, because prolonged sleep deprivation leads to several behavioral and physiological changes, such as changes in body temperature, body weight, food consumption and energy expenditure (Klingenberg et al., 2012). In addition, secretion of anabolic (growth hormone, prolactin and testosterone) and catabolic (glucocorticoids and catecholamines) hormones is also affected by sleep disturbances (Nedeltcheva and Scheer, 2014). Furthermore, sleep deprivation leads to sympathetic activation and has inhibitory effects on insulin secretion, an effect which promotes insulin resistance (Grassi et al., 2005; Levy et al., 2009). Moreover, increased concentrations of serum and urine norepinephrine and epinephrine, two hormones promoting hyperglycemia, have been reported after sleep deprivation in human subjects (Irwin et al., 1999; Nedeltcheva et al., 2009b; Buxton et al., 2010). It has been also reported in human that sleep deprivation enhances evening and early night cortisol concentrations, i.e., the time when levels are low according to the normal circadian pattern (Leproult et al., 1997; Spiegel et al., 1999; Reynolds et al., 2012). This elevation of evening cortisol is thought to favor morning insulin resistance (Plat et al., 1999). Some animal studies also indicate that chronic sleep disturbance is associated with enhanced glucocorticoid levels in serum (Baud et al., 2013), though others suggest plasma corticosterone remains unchanged after long term sleep deprivation (Barf et al., 2012).

Some human studies also report that acute and short term sleep restriction affects glucose metabolism. One night of sleep deprivation impairs glucose tolerance and reduces insulin sensitivity and plasma glucagon levels in healthy men (Schmid et al., 2009; Donga et al., 2010). We replicated this study in our animal model. Results from our experiments show that 4 h of sleep deprivation severely impairs glucose tolerance in rats. To test the possibility of increased HPA-axis activity, we measured plasma corticosterone concentrations. Since plasma corticosterone levels were not different between sleep deprived and control animals, elevated HPA-axis action cannot be the explanation for the impaired glucose tolerance.

The question that remains is how acute sleep deprivation affects glucose metabolism? The hypothalamic orexin system is involved in the regulation of the sleep-wake cycle and glucose

metabolism (Sakurai, 2007; Kalsbeek et al., 2010a). It has been shown that the activity of orexin neurons in the perifornical area (PF) and dorsomedial hypothalamus (DMH) is under circadian control, orexin neurons showing a higher activity during the wake period, while sleep deprivation enhances this activity (Estabrooke et al., 2001). As discussed in the introduction of this thesis, the SCN clock not only controls sleep/wake and fasting/feeding cycles, but also regulates glucose metabolism through the perifornical orexin system (Kalsbeek et al., 2010a). This idea is supported by the findings of Tsuneki et al. (2012) who showed that orexin acts bidirectionally on glucose homeostasis through the autonomic nervous system. The activated orexin neurons seem to alter the sympathetic or parasympathetic outflow to the periphery, and modulate glucose production and utilization, with high and low doses of orexin elevating and decreasing plasma glucose, respectively. Possibly changes in the orexin system in the hypothalamus due to sleep loss could increase glycemia and insulin resistance.

Serotonin in the brain has also been linked to arousal and regulation of glucose metabolism (Asikainen et al., 1997; Versteeg et al., 2015). Serotonin content in the SCN shows a daily rhythm which follows the daily activity cycle in both nocturnal and diurnal rodents (Poncet et al., 1993; Cuesta et al., 2008). In addition, this rhythm is altered in animals showing abnormalities of glucose metabolism (Luo et al., 1999). Sleep deprivation also triggers serotonin release in the SCN (Grossman et al., 2000), but serotoninergic activation leads to hypoglycaemia in mice (Yamada et al., 1989; Sugimoto et al., 1990). Hence, it appears that serotonin may not be involved in the decreased glucose tolerance in sleep deprived animals.

NPY is another hypothalamic neuropeptide that is involved in the integration of the sleep-wake cycle, feeding and glucose metabolism (Szentirmai and Krueger, 2006; Kalsbeek et al., 2010c; Wiater et al., 2011) . The central action of NPY induces hepatic insulin resistance via activation of sympathetic output to the liver (Kalsbeek et al., 2010c) . The PVN which is a relay center for glucose metabolism receives NPYergic projections from the arcuate nucleus. Therefore, it is possible that the impairment of glucose tolerance observed after sleep deprivation could have been mediated through the enhanced stimulation of hypothalamic NPYergic system. The perspective of our current work would be to design future experiments aimed at investigating whether the effect of sleep deprivation on glucose metabolism is mediated through the hypothalamic orexin and/or NPY system.

It has been reported from many human studies that sleep deprivation modifies the secretion of appetite regulatory hormones and increases the risk of obesity. A number of studies documented decreased levels of leptin and increased levels of ghrelin along with increased

hunger in response to sleep deprivation (Reutrakul and Van Cauter, 2014). Some studies also report an increased neuronal activity in certain brain areas linked to the reward system in response to food intake after sleep deprivation (Benedict et al., 2012; St-Onge et al., 2012). Since sleep deprivation prolongs the period of wakefulness, it has been argued that the calories needed to remain awake for this additional period may counterbalance the increase in appetite and food intake. Markwald et al. (2013) have shown that insufficient sleep indeed increases total energy expenditure. However, calorie intake in the night during sleep deprivation exceeds energy intake needed to maintain energy balance, thus favoring weight gain. In contrast to human studies, chronic sleep deprivation in nocturnal animals leads to attenuation of weight gain, regardless of the methods used to create a sleep debt (Klingenberg et al., 2012). Indeed calorimetric measurements in sleep-restricted rats suggest that daily energy expenditure during sleep deprivation is increased compared to control rats without changes in food intake, thus leading to a negative energy balance (Barf et al., 2012). This discrepancy between human and animal studies may result from physiological inter-species differences or differences in the protocols of sleep deprivation. It is important to mention that animals used for laboratory experiments were all nocturnal, while human subjects are diurnally oriented. Because sleep/wake cycle, fasting/feeding rhythm and most hormonal rhythms, including those of leptin, ghrelin and glucocorticoids, usually show an opposite phase between diurnal and nocturnal species (Kumar Jha et al., 2015) , it is important to understand the physiological differences of the effect of sleep deprivation across the species. The perspective of the current work includes designing such experiments of sleep deprivation in diurnal animals, such as *Arvicanthis ansorgei,* to elucidate the physiological differences of sleep deprivation between human and animal species and/or between diurnal and nocturnal species.

4. Feedback action of sleep deprivation on SCN clock

Loss of sleep not only affects the physiological and metabolic homeostasis, but also has the ability to feedback to the central SCN clock to modulate circadian rhythms in mammals. Circadian rhythms are synchronized to local time by the period and phase resetting properties of the environmental factors. These factors are broadly divided into photic and non-photic cues. Resetting of the master clock by light is mainly mediated by the retinohypothalamic pathway to the SCN, originating from the photosensitive retinal ganglion cells (Moore and Lenn, 1972; Rea, 1998; Hattar et al., 2002). The SCN receives information about non-photic stimuli, including behavioral and metabolic cues, from several brain areas, such as the raphe nuclei and the intergeniculate leaflet (IGL). The *Zeitgeber* properties of photic and non-photic

inputs are well established. Arousal is an important non-photic behavioral cue which feeds back to the SCN clock and influences its photic entrainment. Most of the studies investigating entrainment properties of synchronizing factors other than light have been conducted in the usual laboratory rodents, i.e., predominantly nocturnal species. Therefore, only sparse information is available regarding non-photic entrainment in diurnal species. Considering that the temporal organization of sleep and arousal is reversed between nocturnal and diurnal species with respect to the light and dark periods, in Chapter 4 we asked the question whether sleep deprivation and caffeine-induced arousal are synchronizing signals in diurnal animals and, if yes, how they influence photic entrainment when applied during the same astronomical periods as in nocturnal animals (i.e., daytime) or during the same phase of the sleep/wake cycle (i.e., sleep). Our data show that sleep deprivation in the early subjective night in the diurnal Sudanian grass rat, *Arvicanthis ansorgei* induces phase-delays, whereas both sleep deprivation and caffeine treatments enhance light-induced phase-shifts of locomotor activity rhythm. We also investigated the cellular responses of these behavioral changes and found a potentiation of light-induced c-FOS expression in SCN cells, in particular those containing calbindin. Interestingly, these findings are evidently opposite to the results obtained earlier in nocturnal laboratory rodents, such as mice and hamsters.

Is the phase-response curve of arousing stimuli similar in nocturnal and diurnal species?

Unlike the photic phase-response curve (PRC) which has a similar shape in nocturnal and diurnal animals, the non-photic PRC differs in shape between these two species (Challet, 2007). In nocturnal species, the PRC for arousing stimuli shows that both magnitude and direction of the shifts are gated by specific circadian phases, with maximal phase-advances during the middle of the rest period and minimal phase-delays during the latter half of usual wake phase (Hughes and Piggins, 2012; Webb et al., 2014). These behavioral arousals include: induced locomotor activity, sleep deprivation by gentle handling and the mimicking of sleep deprivation by treatment with an adenosine A1 agonist (Yamada et al., 1986; Reebs and Mrosovsky, 1989a; Antle and Mistlberger, 2000; Antle et al., 2001). However, some of the arousing procedures, like caffeine and modafinil treatment, are unable to induce phase-shifts in mice and hamsters, though caffeine does attenuate the phase-shifts induced by sleep deprivation (Antle et al., 2001; Vivanco et al., 2013). In our study in diurnal rodents, we found that sleep deprivation in the early night induced phase-delays, whereas sleep deprivation in late night did not induce significant phase-shifts. These findings are consistent with human studies showing that nocturnal physical activity leads to phase-delays of the circadian rhythm (Buxton et al., 1997). We also studied the effect of caffeine treatment in the

early morning, mid-day, early night and mid-night. In accordance with previous findings in nocturnal species, we found that caffeine on its own is unable to induce phase-shifts of locomotor activity rhythm. However, a recent study in human subjects suggests that caffeine ingestion during the early night does induce phase-delays under dim light condition (Burke et al., 2015). Together, these results show that the phase-response curve to arousal seems oppositely phased in nocturnal and diurnal species as it may depend on the relative phasing of the activity-rest cycle. In order to further strengthen this argument, additional experiments in diurnal animals are needed with other procedures for behavioral arousal, such as induced locomotor activity and treatment with adenosine A1 agonists.

Modulation of photic entrainment by arousal

In laboratory conditions, we aim for rigorously controlled conditions in order to study a particular stimulus, though in nature many *Zeitgebers* interact and act together. Therefore it is important to study the interactions of different *Zeitgebers*. In this section, we discuss how arousing stimuli modulate the light-induced phase-shifts in nocturnal and diurnal animals. Serotonergic cues, which are associated with arousal, have been shown to exert inhibitory effects on photic entrainment in the nocturnal species. On the other hand, serotonergic activation potentiates light-induced phase-shifts of locomotor activity rhythm in the diurnal grass rat, *Arvicanthis ansorgei* (Challet, 2007; Cuesta et al., 2008). Other studies in nocturnal mice and hamsters suggest that different kinds of arousing stimuli, including sleep deprivation by gentle handling, forced physical activity and treatment with an adenosine A1 agonist, have inhibitory effects on light-induced phase-shifts (Mistlberger et al., 1997). A recent study indicates that sleep deprivation in mice reduces the SCN response to the light because it reduces the light-induced firing rate of SCN, while treatment with the adenosine antagonist caffeine rescued this response (van Diepen et al., 2014).

Caffeine treatment has been shown to attenuate light-induced phase-shifts in mice (Vivanco et al., 2013) and similar results have been obtained after treatment with other wake-promoting drugs such as methamphetamine and modafinil (Moriya et al., 1996; Ono et al., 1996; Vivanco et al., 2013). The results of our study indicate that both sleep deprivation and caffeine-induced arousal potentiate light-induced phase-shifts in diurnal grass rats. However, early night caffeine intake in humans did not significantly potentiate the light-induced phase-delay of melatonin secretion (Burke et al., 2015), though the authors argued that this could be due to the saturation effects of light so that the caffeine effect would remain shielded as they used bright light in their experiments. It would be interesting to study how caffeine treatment modulates phase-shifts induced with moderate light intensities, because evening caffeine

treatment induced phase-delays in dim light in that same study. Together, these studies suggest that behavioral arousal interacts with photic entrainment differently in nocturnal and diurnal species. In nocturnal species photic entrainment is slowed down by arousing stimuli, whereas in diurnal species it is accelerated by similar stimuli (**Figure 3**).
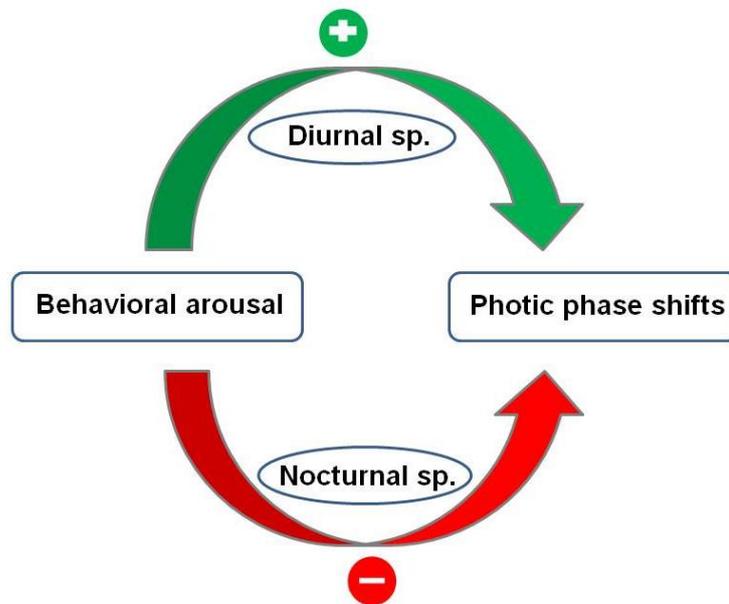

Figure 3. Excitatory and inhibitory effects of behavioral arousal on light-induced phase shifts in diurnal and nocturnal species, respectively

As a future perspective of this study, we need to study the effect of other procedures of arousal on photic entrainment in diurnal rodents, using for instance treatment with adenosine agonists to mimic sleep deprivation, forced locomotor activity and treatment with other wake-promoting pharmaceuticals, like modafinil and methamphetamine.

Pathways and neurotransmitters involved in feedback action of arousal on SCN

As described in the introduction of this thesis, two major SCN afferent pathways are mainly involved in circadian clock resetting by non-photic stimuli such as behavioral arousal. These pathways include the GHT, which originates from the IGL and uses the neurotransmitters NPY and GABA, and projections from the median and dorsal raphe via the IGL to the SCN, using serotonin as a neurotransmitter (Hughes and Piggins, 2012). A third pathway may involve orexin that is critical for the appropriate control of the arousal, via direct projections from the LH to the SCN or indirectly through IGL and median raphe to the SCN. The IGL also receives photic inputs due to its location in visual thalamus and it is activated by light cues, though the NPYergic cells do not show strong activity in response to light exposure (Janik et al., 1995; Grosbellet et al., 2015). It has been shown in hamsters that microinjections

of NPY in the SCN attenuate light-induced phase-advances (Weber and Rea, 1997) . Further experiments with NPY treatments are needed to investigate whether this mechanism is also operative in diurnal rodents. Serotonergic transmission has also been proposed as a potential mediator of phase-shifts induced by arousal and its interaction with photic synchronization in the nocturnal animals (Moriya et al., 1996; Watanabe et al., 1996; Grossman et al., 2000; Challet et al., 2001). In the present study, we did not observe any significant increase in serotonin content in the SCN of either sleep-deprived or caffeine-treated diurnal grass rats. However, midbrain serotonin levels were significantly increased after the caffeine treatment. Previous studies have shown that serotonergic activation in this diurnal animal not only leads to phase-shifts in the subjective night, but also enhances light-induced phase-shifts, two observations that clearly contrast the earlier findings in nocturnal rodents (Challet, 2007; Cuesta et al., 2008). Thus the serotonergic projections from the raphe to the SCN might mediate the stimulatory effects of arousal and sleep-deprivation on light-induced phase-shifts in diurnal species. Nevertheless, further experiments using 5-HT microdialysis in the SCN area could provide relevant information regarding 5-HT release in that region during sleep deprivation and caffeine treatment.

In Syrian hamsters, sleep deprivation and physical exercise elevate c-Fos expression in orexin-expressing cells (Webb et al., 2008). In addition, it has been shown that orexin acutely alters both SCN and IGL neuronal activity *in vitro* (Klisch et al., 2009; Pekala et al., 2011). These findings suggest that increased activity of orexin positive cells in the LH may be correlated with the characteristic non-photic phase-shifts of daytime-induced arousal in nocturnal rodents. Also in the diurnal grass rat, arousal seems to trigger activation of orexin neurons in the LH (Castillo-Ruiz et al., 2010). The present studies did not cover this aspect of arousal-induced phase-shifts, but it would be interesting to know whether the orexin system acts differently in nocturnal and diurnal rodents.

Like serotonergic and orexinergic pathways, the adenosinergic system is also involved in the regulation of behavioral state and circadian rhythms in the animals. The extracellular level of adenosine increases in the CNS after prolonged wakefulness (Huang et al., 2014). It has been discussed earlier that activation of adenosine A1 receptors induces behavioral phase-shifts and attenuates light-induced phase-shifts like other arousing stimuli (Antle et al., 2001; Sigworth and Rea, 2003). Surprisingly, the adenosine antagonist caffeine does not show chronobiotic efficacy, albeit it attenuates light-induced phase-shifts in nocturnal rodents (Antle et al., 2001; Vivanco et al., 2013). Adenosine receptors have been found to be expressed in the SCN, though their involvement in behavioral phase-shifts are not clearly understood (Chen and van

den Pol, 1997; Hallworth et al., 2002). Ryanodine receptors (RyRs) are caffeine-sensitive Ca^{2+} release channels (McPherson et al., 1991) and caffeine treatment may also exert its effect on photic resetting by activating ryanodine receptors (RyRs) in the SCN (Diaz-Munoz et al., 1999). Caffeine-mediated increases in Ca^{2+} levels in SCN neurons were shown to be dependent on the activation of RyRs (Pfeffer et al., 2009). Application of caffeine to mouse SCN slices produces photic like shifts, similar to phase-shifts induced by glutamate. These findings indicate that behavioral arousal induced by sleep deprivation or caffeine treatment may reset the SCN clock by activation of the adenosinergic system in the SCN. However, we did not find any difference in SCN and midbrain adenosine levels between sleep-deprived and control animals, thus suggesting that in diurnal animals this pathway may not be involved. Together, these studies indicate that the feedback action of behavioral arousal to the master clock may act differently in nocturnal and diurnal species. In order to understand better the neurochemical mechanisms specifically involved in diurnal rodents, further experiments with NPY, 5-HT, adenosine and orexin are needed to study their behavioral phase-shifts and their light responsiveness in diurnal species.

Cellular and molecular mechanisms

Arousing stimuli modify immediate early genes expression in the circadian system. In nocturnal animals, behavioral arousal decreases the expression of c-FOS in the SCN and increases it in the IGL (Antle and Mistlberger, 2000) and light-induced c-FOS and p-ERK expression in the SCN of nocturnal rodents is attenuated by the activation of adenosine A1 receptors (Watanabe et al., 1996; Sigworth and Rea, 2003). Similar results have been obtained after treatment with wake-promoting drugs, such as methamphetamine (Moriya et al., 1996; Ono et al., 1996). Our data show that sleep deprivation in the early subjective night increases light-induced c-FOS induction in the ventral SCN in the diurnal grass rat. Moreover, caffeine treatments in the early and mid-subjective-night induce c-FOS expression in the dorsal part of the SCN and also potentiate light-induced expression of c-FOS in the ventral SCN. These findings show that feedback effects of arousal on SCN cellular activity and their interaction with light-induced expression of immediate early genes are opposite in nocturnal and diurnal rodents. The effects on immediate early genes are interesting, but do not provide further insight of clock regulation. Therefore, we performed double immunolabelling of c-FOS with calbindin. The results indicate that both sleep deprivation and caffeine treatment activate a large proportion of the calbindin-containing cells in the SCN of diurnal rodents. In nocturnal animals, it has been shown that SCN calbindin is involved in the maintenance of circadian rhythms and photic entrainment (Hamada et al., 2003; Kriegsfeld et al., 2008; Stadler et al.,

2010), but the effects of sleep deprivation are not known. Our results are the first to suggest an involvement of SCN calbindin-containing cells in the feedback effect of arousal on the master clock and its light-induced phase-shifts, at least in diurnal rodents.

The rhythmic expression of the network of core clock genes and their proteins, including *Period* (*Per1* and *Per2*), *Cryptochrome (Cry 1* and *Cry2)* and *Bmal1,* constitutes the molecular clock in the cell autonomous pacemaker. The expression of most of the clock genes shows a similar pattern in nocturnal and diurnal species. For example, *Per1* in the SCN peaks around mid-day in both species (Challet, 2007). In the clock machinery *Per* genes are the main targets for stimuli that phase-shift the clock. The levels of mRNA of *Per1* and *Per2* are rapidly down-regulated in the SCN by behavioral arousal in nocturnal animals (Webb et al., 2014). It has also been shown that knocking down the *Per1* levels with antisense oligonucleotides during the day induces phase-shifts similar to mid-day arousal (Hamada et al., 2004). A similar decrease in *Per1* and *Per2* expression occurs after serotonergic activation (Horikawa et al., 2000). This decrease in mRNA could lead to a premature rise in clock gene expression in the subsequent cycle, yielding to the observed phase-advance. Contrary to nocturnal animals, serotonergic activation in diurnal grass rats does not change *Per* expression, suggesting different molecular mechanisms that remain to be identified (Cuesta et al., 2008). Differences in the window of sensitivity for serotonergic activation between nocturnal and diurnal species could be a possible explanation. Whether behavioral arousal itself affects *Per* expression in the diurnal rodents still remains a matter of investigation. The perspective of our current investigation includes to study in future experiments expression of *Per* and other clock genes after sleep deprivation or caffeine treatment in diurnal grass rats.

General conclusion

The present thesis investigated how sleep loss affects metabolic homeostasis and circadian rhythms in nocturnal and diurnal species. By investigating reduced sleep duration and its consequences on energy metabolism and physiology, we addressed a question which is relevant in a larger framework to the modern society in which voluntary sleep curtailment is prevalent.

Short sleep duration has been associated with the increased prevalence of type 2 diabetes, obesity and cardiovascular abnormalities. Increasing evidence suggests sleep plays an important role in the regulation of glucose metabolism. Disruptions in the sleep-wake cycle may have deleterious effects on glucose metabolism. As discussed earlier, plasma glucose concentrations and glucose tolerance are highest in the morning and lowest in the evening in humans, while these parameters are oppositely phased in the nocturnal species (Kumar Jha et

al., 2015). One question raised by these oppositely phased rhythms is whether the SCN employs similar or different neural mechanisms to control the rhythms in plasma glucose concentration, glucose tolerance and insulin sensitivity. The sleep-wake rhythms in the nocturnal and diurnal species are oppositely phased according to the astronomical light-dark cycle, but the SCN clock is active at the same astronomical time in these two species (Smale et al., 2003) . Experiments in rats have established that the SCN clock regulates glucose metabolism through different hypothalamic nuclei and neuropeptides and ultimately the autonomic nervous system (Kalsbeek et al., 2010c). As an extension of these investigations, the question was raised in Chapter 2 how GRP, another SCN neuropeptide, affects glucose metabolism. Our results show that central treatment of rats with the SCN neuropeptide GRP induces immediate hyperglycemia by enhancing EGP and glucagon release. Possibly SCN-mediated activation of the GRP-R leads to an increase in plasma glucose concentrations and rhythmic release of GRP may contribute in regulation of glucose homeostasis (**Figure 4**). It would be interesting to perform similar experiments in diurnal grass rat*s* to understand how the SCN controls glucose metabolism in the diurnal species.
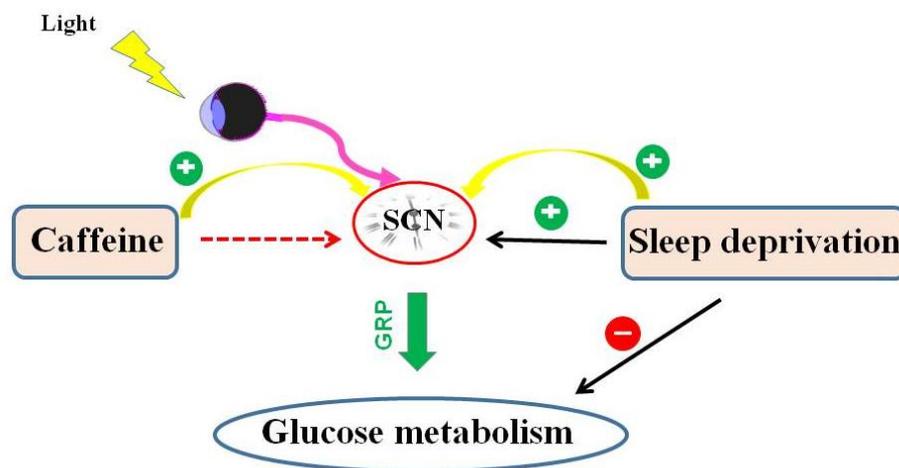
Figure 4. Effect of sleep deprivation and caffeine treatment on clock resetting and glucose metabolism

The SCN may employ GRP to control glucose metabolism. Sleep deprivation affects glucose metabolism through impairment of glucose tolerance in rats. Acute treatment of caffeine does not affect glucose tolerance in rats. Sleep deprivation induces phase delays. Caffeine treatment does not induce phase shifts, but both sleep deprivation and caffeine potentiate light-induced phase shifts in diurnal rodent, *Arvicanthis ansorgei*.

During the last 15 years, a large number of epidemiological studies and experiments in human subjects have documented that insufficient sleep leads to weight gain and abnormalities in glucose metabolism. However, this finding has not been clearly replicated in experimental animals. In an attempt to further our understanding of the impact and mechanism of acute

sleep deprivation on glucose metabolism, we studied how short sleep deprivation affects glucose tolerance in rats. Our results show that only 4 h of sleep deprivation in the early resting period or middle of the rest period severely impairs glucose tolerance in rats. Taken together, these studies further support the notion of a good night's sleep as a crucial factor for normal metabolic functioning. Thus, appropriate duration and timing of sleep represents an important therapeutic recommendation for the prevention or limitation of the global epidemic increase in obesity and T2D.

Whereas chronic sleep loss has been proven deleterious for metabolic and physiological health, acute sleep deprivation has been shown to have antidepressant effects (Wirz-Justice and Van den Hoofdakker, 1999). Severe depressions are accompanied with abnormalities of circadian rhythms which include sleep, hormonal and body temperature dysregulation. It has been hypothesized that the circadian machinery may be reset by the clock resetting properties of arousal, which eventually would alleviate the depressive symptoms (Bunney and Bunney, 2013). In addition to sleep deprivation, bright light therapy in the morning has also been used as an antidepressant treatment, because light is an important *Zeitgeber* and morning bright light induces phase-advances of circadian rhythms (Wirz-Justice et al., 2005). The clock resetting action of behavioral arousal has been studied extensively in nocturnal rodents. Our experiments aimed to study the feedback action of sleep deprivation and caffeine induced arousal on the SCN clock and its interaction with photic entrainment in the diurnal grass rat, *Arvicanthis ansorgei,* in order to obtain a better insight in the difference between nocturnality and diurnality. We showed that sleep deprivation induces phase-delays and it enhances the light-induced phase-shift. In addition, caffeine treatment was unable to produce significant phase-shifts, but like sleep deprivation, it also potentiated photic resetting (**Figure 4**). Further experiments in other diurnal species are needed to generalize these findings to diurnality. A previous study in humans has also reported that nocturnal physical activity induces phase-delays (Buxton et al., 1997). Moreover, the fact that arousing stimuli caused by sleep deprivation and caffeine treatment have the potential to enhance light synchronization may add its relevance to biomedical applications. Both sleep deprivation and caffeine treatment could increase the sensitivity of depressed patients to the synchronizing cues of light during phototherapy. Because the modulation of circadian rhythms by behavioral arousal appears different between nocturnal and diurnal species, more chronobiological studies are required in diurnal species in order to better understand diurnality and to develop innovative and more efficient chronotherapeutic treatments in humans.

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APPENDICES

Summary

Samenvating

Résumé

Thesis abstract

PhD Portfolio

Acknowledgements

SUMMARY

Circadian rhythms in nocturnal and diurnal mammals are controlled by a light-entrainable circadian pacemaker, in the suprachiasmatic nuclei (SCN) of the hypothalamus, which is primarily synchronized to local time by the light-dark cycle. The SCN controls behavioural and metabolic rhythms by relaying timing information to peripheral tissues. For instance, daily rhythms of sleep-wake, fasting-feeding, plasma glucose concentration, glucose tolerance and insulin sensitivity are regulated by the SCN clock.

In order to maintain glucose homeostasis, the SCN transmit their timing signals via the autonomic nervous system through hypothalamic relay structures, such as the hypothalamic paraventricular nucleus (PVN) and the orexin-containing neurons in the perifornical area (PF). Rhythmic excitatory and inhibitory signals from the SCN to these extra SCN nuclei help to maintain daily rhythm of plasma glucose concentration via sympathetic inputs to the liver. Neuropeptides other than orexin have also been implicated in the hypothalamic control of glucose homeostasis. A number of neuropeptides such as arginine vasopressin (AVP), vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) show a daily rhythm in their release pattern from the SCN. These neuropeptides might communicate time-cues to metabolic organs via nervous and humoral signals in order to regulate the daily rhythmicity of metabolic processes. Possibly these neuropeptides are also involved in SCN control over glucose homeostasis. GRP-mediated signals influence many functions in the central nervous system, including food intake, body weight, glucose metabolism and circadian rhythms. In the present thesis we investigated the possible involvement of GRP in the control of plasma glucose homeostasis (Chapter 2). We showed that intracerebroventricular (i.c.v) administration of GRP caused an immediate hyperglycaemia which was sustained till the end of the infusion. The rise in plasma glucose levels was accompanied by an increase in endogenous glucose production (EGP), as well as increases in plasma glucagon and insulin concentrations. Furthermore, no differences in plasma corticosterone levels were noted between control and GRP treated rats. SCN-mediated rhythmic excitation and inhibition of pre-autonomic neurons in the PVN helps to maintain daily glucose homeostasis through the autonomic inputs to the liver. GRP content in the rat SCN increases during the day and gradually decreases during the night. Moreover, GRP receptor (GRP-R) and its binding show daily rhythms with an acrophase in early night similar to the plasma glucose rhythm, though it is not known whether GRP-R expression in PVN is rhythmic or not. Central treatment of GRP

enhances c-FOS expression in SCN and PVN. However, it is not clear which cells in SCN and PVN got activated by the GRP treatment. Based on these results we hypothesized that the GRP-induced hyperglycemia may involve an activation of PVN neurons by GRP resulting in increased sympathetic inputs to the pancreas and liver, thus leading to subsequent increased glucagon release and EGP.

Plasma glucose concentrations follow a daily rhythm which is anti-phasic between nocturnal and diurnal rodents. There is no evidence that SCN neuropeptides vary in opposite phase in nocturnal and diurnal species. Though, the release pattern of most of these SCN neuropeptides in nocturnal rodents is not known in detail and has not been studied in diurnal animals at all. In order to understand the GRP-mediated regulation of glucose homeostasis in nocturnal and diurnal species, we need to perform this experiment in time of the day dependent manner in both nocturnal rats and diurnal rodents, such as *Arvicanthis ansorgei*.

The SCN not only control plasma glucose rhythm, but they also regulate the rhythm of glucose utilization. Both in human and animal studies, glucose tolerance is highest at their activity onset, i.e., morning and evening in diurnal humans and nocturnal rodents, respectively. Increasing evidence from epidemiological as well as experimental studies indicate that reductions in sleep duration may result in increased risks of obesity, type 2 diabetes and cardiovascular abnormalities. Earlier human studies showed that reduced sleep duration and quality impair glucose tolerance. In our study, we investigated the effect of acute short-term sleep restriction on glucose tolerance in rats (Chapter 3). A group of animals was sleep deprived for 4 h in early resting period and another group was sleep deprived in the middle of the rest period. Immediately after the sleep deprivation period, we performed intravenous glucose tolerance tests (IVGTTs). The results of these studies indicate that 4 h of sleep deprivation severely impairs glucose tolerance as assessed by IVGTTs and reduces the early-phase insulin responses to glucose loads, irrespective of their time window, i.e., early or middle rest period. Basal concentrations of plasma glucose and insulin remained unchanged before and after sleep deprivation. We also found that plasma levels of corticosterone were not different between the control and sleep-deprived groups, which ruled out a possible activation of the hypothalamo-pituitary-adrenal-axis as an explanation for the impaired glucose tolerance during IVGTT. These results suggest that independently of time-of-day and sleep pressure, acute short sleep deprivation during the resting phase induces glucose intolerance in rats by attenuating the first-phase insulin response to a glucose challenge.

The reduced glucose disposal in sleep deprived animals during IVGTTs may result from a high glucose production or its reduced uptake. Our present data could not differentiate

whether observed hyperglycemia is because of reduced glucose uptake or high glucose production. The reduced early insulin response in the sleep deprived groups will result both in a reduced glucose uptake as well as in a lesser inhibition of glucose production. Experiments using the stable isotope dilution technique to determine endogenous glucose production (EGP) need to be done to understand the nature of observed hyperglycemia in sleep deprived animals during IVGTTs. It has been reported earlier that sleep deprivation activates the sympathetic branch of the autonomic nervous system and release of catecholamines in the general circulation. The reduction in the early-phase insulin response to glucose load could be associated with enhanced sympathetic and/or reduced parasympathetic activity. Now the remaining question is: what could be the central structures mediating the observed peripheral effects of sleep deprivation? We hypothesize that a likely candidate is the hypothalamic orexin system, because of its involvement in the regulation of the sleep/wake cycle and the daily rhythm of glucose metabolism. Another putative candidate is the hypothalamic NPY system because of its involvement in regulation of arousal and glucose metabolism.

We need to validate whether the observed effect of sleep deprivation on glucose metabolism is mediated through the hypothalamic orexin and/or NPY system. Moreover, sleep-wake cycle is oppositely phased in nocturnal and diurnal species according to the astronomical light/dark cycle, while plasma glucose concentrations also show oppositely phased rhythms between nocturnal and diurnal rodents. Therefore, it would be interesting to perform similar experiments in the diurnal rodents and to test whether sleep deprivation alters glucose metabolism similarly or differently. Unravelling the mechanisms of alteration in glucose homeostasis due to sleep restriction are clearly relevant for biomedical application.

Sleep deprivation and other procedures of behavioural arousal also have the ability to reset the phase and period of the master pacemaker, involving signals from other brain areas to the SCN. It has been established that behavioural arousal induces phase shifts and reduces light-induced phase-shifts of locomotor activity rhythm in nocturnal rodents. In these animals, activation of the serotonergic pathways produces phase-shifts during daytime and attenuates the phase-shifting effects of light on the circadian pacemaker at night. On the other hand, in diurnal animals, activation of the serotonergic pathways produces phase-shifts at night and enhances the phase-shifting effects of light. Thus, the role of serotonergic feedback system seems to act oppositely in nocturnal and diurnal animals. Sleep deprivation in nocturnal rodents has been shown to shift the clock during daytime and to decrease the phase-shifting effects of light at night. In diurnal rodents, however, the role of arousal or sleep deprivation in these functions has not been studied intensively yet. This prompted us to study the feedback

action of induced behavioural arousal on SCN clock and light-induced phase-shifts in diurnal animals. We addressed the question of how sleep deprivation and caffeine-induced arousal affect the SCN clock and its light sensitivity in a diurnal rodent, the Sudanian grass rat, *Arvicanthis ansorgei* (Chapter 4). Phase-shifts of locomotor activity were analysed in grass rats transferred from a light-dark cycle to constant darkness and aroused in early night (sleep deprivation from Circadian Time 12 (CT12) to CT16 or caffeine injection at CT14) and late-night (sleep deprivation from CT16 to CT20 or caffeine injection at CT18) and/or exposed to a 30-min light pulse at CT16 and CT20, respectively. The analysis of phase shifts shows that sleep deprivation induced significant phase-shifts (delays) and potentiated light-induced phase-delays and phase-advances in early and mid-subjective night, respectively. Sleep deprivation in early night potentiated light-induced c-FOS expression in the ventral SCN. We also phenotyped these neurons by selecting calbindin as a putative candidate. Sleep deprivation in early and late-night increased c-FOS expression in calbindin-containing cells in the SCN. Furthermore, early and mid-subjective night caffeine treatments did not induce significant shift, but potentiated light-induced phase-delays and phase-advances, respectively. Caffeine treatment in early and mid-night potentiated light-induced c-FOS expression in the ventral SCN and mid-night treatment induces c-FOS expression in dorsal SCN. Again, we phenotyped these neurons by selecting calbindin as a putative candidate. Caffeine treatment in early- and mid-night increased c-FOS expression in calbindin-positive cells in the SCN.

We also assessed the levels of serotonin (5-HT) and adenosine in SCN and midbrain after sleep deprivation and caffeine treatment during early subjective night (CT 14). The levels of 5-HT were not different in SCN and midbrain after sleep deprivation. However caffeine treatment enhanced 5-HT levels in the midbrain after caffeine treatment. Adenosine concentrations in the SCN and midbrain did not change after sleep deprivation and caffeine treatment.

Previous studies in nocturnal rodents indicated that sleep deprivation or forced locomotor activity in the middle of rest period induce large phase-advances shifts. Treatment with adenosine A1 receptor agonist in middle of rest period, a procedure aimed to mimic sleep deprivation pharmacologically also induces phase-advance shift in a dose-dependent manner. It has been shown from series of experiments that the phase-response curves (PRC) of behavioural arousal are similar to a typical non-photic PRC having maximal phase-advances during the middle of the rest period and minimal phase-delays during the latter half of the usual wake period. In present study, we show that early night sleep deprivation in the diurnal grass rat, *Arvicanthis ansorgei* induces phase-delays, however, sleep deprivation in late night

did not induce significant shifts compared to undisturbed controls. Like previous studies in nocturnal mice and hamsters, caffeine alone could not induce phase-shift. These findings indicate that shape and timing of phase-response to arousing stimuli depend on the rest-activity cycle which is opposite between nocturnal and diurnal species.

Arousing stimuli have been shown to interact with the photic entrainment. Acute sleep deprivation (e.g., gentle handling), forced activity, or injections of an adenosine A1 agonist inhibit light-induced phase-shifts in nocturnal rodents. Surprisingly, the adenosine antagonist caffeine also blocks light-induced phase-delays in the mice like other wake promoting molecules such as methamphetamine and modafinil. Our data show that both sleep deprivation and caffeine treatment potentiated light-induced phase-delay and advance shifts in a diurnal rodent. This result suggests that behavioural arousal, induced by sleep deprivation or caffeine treatment, differentially affects photic resetting of the circadian master clock in nocturnal and diurnal rodents

Light-induced c-FOS expression in the SCN, which is similar between nocturnal and diurnal rodents, has been used as a functional marker. It has reported earlier that behavioural arousal reduces c-FOS expression in the SCN and reduces light-induced c-FOS and p-ERK expression in the SCN of nocturnal rodents. Our results show that both sleep deprivation and caffeine treatment increase light-induced c-FOS induction in the ventral SCN, indicating that feedback effects of arousal on SCN cellular activity and their interaction with the light-induced expression of immediate early genes are opposite in nocturnal and diurnal rodents. We also phenotyped these neurons as being calbindin-containing cells in both sleep-deprived and caffeine-treated animals.

Increased concentrations of serotonin in the SCN and other hypothalamic areas have been correlated with the circadian phase shifts and/or attenuated light-induced phase-shifts observed after sleep deprivation in nocturnal rodents. In addition, wake-promoting pharmaceuticals, like methamphetamine, also increase the release of serotonin in the SCN. Our results suggest that 4 h of sleep deprivation did not change serotonin contents in the SCN and midbrain. By contrast, caffeine treatment enhances serotonin in midbrain. There is evidence of presence of adenosine receptors in the SCN. At the same time, microinjections of adenosine agonist in the SCN of hamsters and rats induce phase-shifts, as does sleep deprivation, and these compounds attenuate light-induced phase-shifts. However, our data show no difference in adenosine content in SCN and midbrain after early-night sleep deprivation and caffeine treatment. Therefore, it seems that arousal feedback in diurnal

rodents follows pathways other than the serotonergic and adenosinergic ones (e.g., orexinergic cues).

Conclusion

The current thesis deals with different aspects of interaction between sleep deprivation, circadian clock and metabolism in nocturnal and diurnal species. In the second chapter of this thesis, we extended the investigation of SCN control of glucose metabolism by asking the question of how central action of GRP affects glucose homeostasis. Using icv infusions of GRP and the stable isotope dilution technique to determine endogenous glucose production (EGP), we show that central action of GRP leads to hyperglycemia accompanied by an increase in EGP, as well as increases in plasma glucagon and insulin concentrations. Therefore, this experiment demonstrates that GRP-induced hyperglycemia is probably due to pancreatic glucagon release and/or EGP. Further studies are needed to understand whether this effect is mediated by the SCN or not. Glucose homeostasis is also affected by sleep/wake alterations, including sleep deprivation. In the third chapter, we investigated the effects of acute short term sleep deprivation on glucose tolerance in rats. Results of this study indicate that insufficient sleep leads to impairment of glucose tolerance, which is considered as a pre-diabetic condition. Since the plasma glucose concentration and expression of GRP in the SCN show anti-phasic rhythms between nocturnal mice and diurnal the diurnal grass rat, *Arvicanthis ansorgei*, it will interesting to investigate whether the effects of central GRP and sleep deprivation on glucose metabolism also depend on the time-of-day and the nocturnal/diurnal nature of a species.

In the fourth chapter we investigated the question of how behavioural arousal affects the functioning of circadian clock in the diurnal rodent, *Arvicanthis ansorgei*. Various studies in nocturnal rodents have documented that behavioural arousal provides feedback cues to the SCN by shifting the master clock during daytime and reducing photic resetting. Contrary to what is observed in nocturnal rodents, we show that sleep deprivation in early rest period induces phase-delays in this diurnal rodent. Arousal induced either by sleep deprivation or caffeine treatment potentiates photic resetting in part by activating calbindin-containing cells of the SCN and without involving serotonergic and adenosinergic systems. Unlike nocturnal rodents, diurnal rodents do not show photosomnolence and instead, they display increased alertness to bright light exposure, as humans. Therefore, it is important to address further the question of circadian impairments including shift work and trans-meridian travel in this rodent model.

SAMENVATING

Dag/nacht ritmes in zoogdieren, zowel nacht-actieve als dag-actieve, worden gereguleerd door een lichtgevoelige circadiane pacemaker in de nucleus suprachiasmaticus (SCN) van de hypothalamus. Deze interne pacemaker blijft gesynchroniseerd met de lokale tijd in de buiten wereld voornamelijk via de afwisseling van licht en donker. De SCN reguleert zowel gedrags- als metabole ritmes door deze tijd informatie door te geven aan de perifere weefsels. Het dagelijkse slaap/waak ritme, maar bijvoorbeeld ook de dagelijkse ritmes in vasten/voeden, plasma glucose concentratie, glucose tolerantie en insuline gevoeligheid worden gereguleerd door de SCN klok.

Om glucose homeostase te bewaren geeft de SCN haar tijdsignalen door aan het autonome zenuwstelsel via hypothalame tussenstations zoals de nucleus paraventricularis (PVN) en de orexine-bevattende neuronen in het perifornicaal gebied (PF). Ritmische signalen vanuit de SCN, zowel stimulerend als remmend, naar deze hersengebieden en vervolgens de sympathische innervatie van de lever zorgen voor een dagelijks ritme in plasma glucose concentraties. Naast orexine zijn ook andere neuropeptiden mogelijk betrokken bij deze hypothalame regulatie van de glucosehuishouding. Verschillende neuropeptiden, zoals bijvoorbeeld arginine vasopressine (AVP), vasoactief intestinaal peptide (VIP) en gastrine-releasing peptide (GRP), vertonen een dag/nacht ritme in de SCN. Deze neuropeptiden zouden dus via nerveuze of hormonale signalen tijd informatie kunnen doorgeven aan metabole organen om dagelijkse ritmes in metabole processen te reguleren en mogelijk zijn deze neuropeptiden dus ook betrokken bij de SCN regulatie van de glucosehuishouding. GRP signalen mediëren vele functies in het centrale zenuwstelsel, zoals eetgedrag, lichaamsgewicht, glucose metabolisme en circadiane ritmes. In dit proefschrift hebben we de mogelijke betrokkenheid van GRP bij de regulatie van het dag/nacht ritme in plasma glucose onderzocht (Hoofdstuk 2).

We konden aantonen dat de intracerebroventriculaire (i.c.v.) toediening van GRP resulteerde in een onmiddellijke verhoging van de plasma glucoseconcentratie die aanhield tot het einde van de i.c.v. toediening van GRP. De stijging van plasma glucose ging gepaard met een stijging van de endogene glucose productie (EGP), als mede stijgingen van de plasma glucagon en insuline concentraties. Plasma corticosteron concentraties verschilden echter niet tussen de GRP- en controle dieren. SCN-gemedieerde ritmische excitatie en remming van pre-autonome neuronen in de PVN is, via de autonome innervatie van de lever, betrokken bij de regulatie van het dagelijkse glucose ritme. De hoeveelheid GRP in de SCN stijgt gedurende

de dag en neemt langzaam weer af gedurende de nacht. Bovendien vertoont ook de hoeveelheid GRP-receptor (GPR-R) en receptor binding een dagelijks ritme met een piek aan het begin van de nacht, vergelijkbaar met het ritme in plasma glucose concentratie. Het is echter niet bekend of ook de GRP-R expressie in de PVN ritmisch is. Centrale toediening van GRP verhoogt de expressie van c-FOS in de SCN en PVN, maar het is nog niet duidelijk welke cellen in de SCN en PVN worden geactiveerd door GRP. Op grond van bovengenoemde resultaten stellen we voor dat de GRP-gemedieerde hyperglycaemie het gevolg is van een activatie van PVN neuronen door GRP welke resulteert in een toegenomen sympathische input naar pancreas en lever en vervolgens in een verhoogde glucagon afgifte en EGP.

Het dagelijkse ritme in plasma glucose concentraties is in anti-fase tussen nacht-actieve en dag-actieve knaagdieren. Er is geen bewijs dat neuropeptiden in de SCN een tegengestelde fase vertonen in dag-actieve en nacht-actieve soorten. Voor de meeste neuropeptiden is dit afgiftepatroon echter slechts gedeeltelijk bekend in nacht-actieve dieren, en in het geheel niet voor dag-actieve dieren. Om de rol van GRP in de regulatie van glucosehomeostase beter te begrijpen in zowel nacht- als dag-actieve dieren moet in toekomstige experimenten het effect van GRP onderzocht worden op verschillende momenten van de licht/donker cyclus, in zowel nacht-actieve ratten als een dag-actief knaagdier, zoals bijvoorbeeld de *Arvicanthis ansorgei*.

De SCN reguleert niet alleen het dagelijkse ritme in plasma glucose, maar ook het dagelijkse ritme in glucosegebruik. Zowel in dierexperimentele als humane studies is glucosetolerantie het hoogst aan het begin van de activiteit periode, d.w.z. respectievelijk 's morgens en 's avonds in dag actieve mensen en nacht actieve knaagdieren.

Meer en meer bewijs uit zowel epidemiologische als experimentele studies wijst er op dat een afname van de slaapduur resulteert in een verhoogd risico op overgewicht, type 2 diabetes en cardiovasculaire problemen. Een aantal eerdere humane studies heeft laten zien dat zowel een verminderde slaapduur als een verminderde slaapkwaliteit glucose tolerantie verslechteren. In onze eigen studie onderzochten we het acute effect van een korte slaapdeprivatie op glucosetolerantie in ratten (Hoofdstuk 3). Eén groep dieren werd 4 uur slaap-gedepriveerd tijdens het begin van de slaapperiode en een tweede groep in het midden van de slaapperiode. Direct na de slaapdeprivatie werd een intraveneuze glucosetolerantietest (IVGTT) uitgevoerd. De resultaten van deze studies geven aan dat 4 uur slaapdeprivatie een sterke verslechtering van de glucosetolerantie tot gevolg heeft alsmede een verminderde vroege-fase insuline afgifte, ongeacht de timing van de slaapdeprivatie, d.w.z. aan het begin of in het midden van de slaapperiode. De basale glucose en insuline concentraties verschilden niet na 4 uur met of

zonder slaap. Ook de plasma corticosteron concentraties waren niet verschillend tijdens de IVGTT's na 4 uur slaap of slaapdeprivatie. Dit laatste maakt het onwaarschijnlijk dat een activatie van de hypothalamus-hypofyse-bijnier-as de oorzaak is van de verminderde glucosetolerantie tijdens een IVGTT na slaapdeprivatie. Deze resultaten geven aan dat een korte slaapdeprivatie, onafhankelijk van de tijd-van-de-dag en de slaapdruk, acuut glucose intolerantie induceert in ratten door het verminderen van de vroege insuline afgifte na een glucose bolus.

De afgenomen glucose verwerking in slaap-gedepriveerde dieren tijdens een IVGTT zou het gevolg kunnen zijn van een verhoogde glucoseproductie of een verminderde glucoseopname. Met de huidige data kunnen we geen onderscheid maken tussen hyperglycaemie als gevolg van een verminderde glucoseopname dan wel een verhoogde glucoseproductie. De verminderde vroege-fase insuline afgifte heeft zowel een verminderde glucoseopname als een verhoogde glucoseproductie tot gevolg. Vervolgexperimenten met de stabiele isotoop techniek om de endogene glucoseproductie (EGP) te meten zijn noodzakelijk om de oorzaak van de hyperglycaemie in slaap gedepriveerde dieren tijdens een IVGTT verder te begrijpen. Eerdere studies hebben gerapporteerd dat slaapdeprivatie de sympathische tak van het autonome zenuwstelsel activeert alsook de afgifte van catecholamines in de algemene circulatie. De verminderde vroege-fase insuline afgifte na een glucosebolus zou het gevolg kunnen zijn van een verhoogde sympathische en/of verminderde parasympathische activiteit. De resterende vraag is dan nog: welke hersengebieden zijn verantwoordelijk voor de waargenomen perifere effecten van slaap deprivatie? Wij stellen het hypothalame orexine systeem voor als een voor de hand liggende kandidaat, gezien zijn betrokkenheid bij de regulatie van de slaap/waak cyclus alsmede het dagelijkse ritme in glucose metabolisme. Een andere mogelijke kandidaat is het hypothalame NPY systeem gezien zijn betrokkenheid bij activiteit/alertheid en het glucosemetabolisme.

Toekomstige experimenten moeten aantonen of de waargenomen effecten van slaapdeprivatie op het glucosemetabolisme gemedieerd worden door het hypothalame orexine en/of NPY systeem.

Ten opzichte van de astronomische dag/nacht cyclus vertoont de slaap/waak cyclus van nacht-actieve en dag-actieve dieren een tegengestelde fase. Ook de plasma glucoseconcentraties vertonen een tegengestelde fase in nacht-actieve en dag-actieve knaagdieren. Een interessant vervolg zou dus zijn om dezelfde experimenten ook uit te voeren in een dag-actief knaagdier om te onderzoeken of slaapdeprivatie glucose metabolisme op dezelfde manier veranderd of anders. Het is duidelijk dat het ontrafelen van het mechanisme van de slaapdeprivatie-

geïnduceerde veranderingen in glucosemetabolisme een duidelijke biomedische relevantie heeft.

Slaapdeprivatie en andere ingrepen die een verhoogde gedragsactiviteit veroorzaken hebben ook de capaciteit om de fase en periode van de master pacemaker te beïnvloeden, door middel van signalen uit andere hersengebieden naar de SCN. Eerdere experimenten hebben laten zien dat in nacht-actieve dieren een verhoogde gedragsactivatie fase-verschuivingen induceert en licht-geïnduceerde fase-verschuivingen van het activiteits ritme (gedeeltelijk) blokkeert. Activatie van het serotonine systeem gedurende de lichtperiode zorgt voor een fase-verschuiving, terwijl tijdens de donkerperiode een dergelijke activatie zorgt voor afnemende licht-geïnduceerde fase-verschuivingen van de circadiane pacemaker. In dag-actieve dieren daarentegen zorgt een activatie van het serotonerge systeem tijdens de donkerperiode voor een fase-verschuiving en versterkt het de licht-geïnduceerde fase-verschuivingen. Het serotonine systeem lijkt dus tegengestelde effecten te hebben in nacht-actieve en dag-actieve dieren. Van slaapdeprivatie is het bekend dat het in nacht-actieve dieren zorgt voor een fase-verschuiving van de klok tijdens de lichtperiode, en een afname van de licht-geïnduceerde verschuivingen tijdens de donkerperiode. In dag-actieve dieren is echter nog weinig bekend over de effecten van gedragsactivatie of slaapdeprivatie op bovengenoemde functies. Reden voor ons om het terugkoppelings effect van gedragsactivatie op de SCN klok en licht-geïnduceerde fase-verschuivingen te onderzoeken in een dag-actief knaagdier.

Wij hebben onderzocht hoe slaapdeprivatie en cafeïne-geïnduceerde gedragsactivatie de SCN klok en zijn lichtgevoeligheid beïnvloeden in een dag-actief knaagdier, de Soedanese gras rat, *Arvicanthis ansorgei* (Hoofdstuk 4). Fase-verschuivingen in het activiteit ritme werden geanalyseerd in Soedanese gras ratten die waren overgebracht van een licht/donker cyclus naar continu donker en vervolgens wakker gehouden aan het begin van de nacht (middels slaapdeprivatie van CT12 tot CT16 of een cafeïne injectie op CT14) of aan het eind van de nacht (slaapdeprivatie van CT16 tot CT20 of een cafeïne injectie op CT18), al dan niet met blootstelling aan een lichtpuls van 30 minuten op respectievelijk CT16 of CT20. De analyse van de fase-verschuivingen laat zien dat slaapdeprivatie een significante fase-verschuiving (vertraging) veroorzaakt en dat het de licht-geïnduceerde fase-vertraging en fase-versnelling aan respectievelijk het begin en eind van de nacht versterkt. Slaapdeprivatie aan het begin van de nacht versterkte ook de licht-geïnduceerde c-FOS expressie in de ventrale SCN. Deze neuronen konden vervolgens worden gefenotypeerd als zijnde calbindin positief. Slaap deprivatie aan het begin en eind van de nacht verhoogde de expressie van c-FOS in calbindin-positieve cellen in de SCN. Cafeïne injecties aan het begin en eind van de nacht

veroorzaakten geen significante fase-verschuivingen, maar versterkten wel de licht-geïnduceerde fase-vertraging en fase-versnelling. De cafeïne behandeling aan het begin en eind van de nacht verhoogde ook de licht-geïnduceerde c-FOS expressie in de ventrale SCN, de behandeling aan het eind van de nacht veroorzaakte ook c-FOS expressie in de dorsale SCN. Ook nu konden we deze neuronen feno-typeren met behulp van calbindin. Cafeïne behandeling aan het begin en eind van de nacht verhoogde de c-FOS expressie in calbindin-positieve cellen in de SCN.

We hebben ook de concentraties van serotonine (5-HT) en adenosine in de SCN en middenhersenen gemeten na de slaapdeprivatie en cafeïne behandeling aan het begin van de nacht. De concentratie van 5-HT in de SCN en middenhersenen was niet veranderd na slaapdeprivatie. Echter, de behandeling met cafeïne verhoogde de concentratie van 5-HT in de middenhersenen. De concentratie van adenosine in de SCN en middenhersenen was niet veranderd na slaapdeprivatie of cafeïne behandeling.

Eerdere studies in nacht-actieve knaagdieren gaven aan dat slaapdeprivatie of gedwongen activiteit in het midden van de rust periode resulteerde in grote fase-verschuivingen. Behandeling met een adenosine A1 receptor agonist in het midden van de rust periode, een behandeling bedoeld om slaapdeprivatie farmacologisch na te bootsen, veroorzaakte ook fase-versnellingen op een dosis-afhankelijke manier. Uit een hele serie van experimenten is het inmiddels duidelijk geworden dat de fase-response curve (PRC) van gedragsactivatie vergelijkbaar is met een typische non-photische PRC, met een maximale fase-versnelling in het midden van de rustperiode en een minimale fase-vertraging tijdens de tweede helft van de gebruikelijke rustperiode. In de huidige studie laten we zien dat slaapdeprivatie in de dag-actieve gras rat *Arvicanthis ansorgei* aan het begin van de nacht een fase-vertraging veroorzaakt, terwijl slaapdeprivatie aan het eind van de nacht geen significante effecten had vergeleken met de controle conditie. Net als eerdere studies in nacht-actieve muizen en hamsters kon cafeïne alleen geen significante fase-verschuivingen induceren. Deze resultaten maken duidelijk dat de vorm en timing van een fase-verschuiving als gevolg van een gedragsactiverende stimulus afhangen van de rust-activiteit cyclus, welke tegengesteld is in nacht-actieve en dag-actieve dieren.

Het is bekend dat gedragsactiverende stimuli ook kunnen interacteren met de photische entrainering. Acute slaap deprivatie (bijv. door voorzichtig hanteren), gedwongen activiteit of injectie van een adenosine A1 agonist remmen de licht-geïnduceerde fase-verschuivingen in een nacht-actief knaagdier. Verrassend genoeg kan in de muis de adenosine antagonist cafeïne licht-geïnduceerde fase-vertragingen ook blokkeren, net als andere activiteit stimulerende

moleculen zoals metamphetamine en modafinil. Onze data laten zien dat zowel slaapdeprivatie als cafeïne licht-geïnduceerde fase-vertragingen en –versnellingen kunnen versterken in een dag-actief dier. Deze resultaten suggereren dat de effecten van gedragsactivatie door slaapdeprivatie of cafeïne op licht-geïnduceerde verschuivingen van de circadiane meester klok verschillen tussen nacht-actieve en dag-actieve dieren.

We hebben licht-geïnduceerde c-FOS expressie in de SCN, die gelijkelijk op treedt in nacht-actieve en dag-actieve dieren, gebruikt als een functionele marker. Eerdere experimenten hebben laten zien dat gedragsactivatie c-FOS expressie in de SCN reduceert, als ook licht-geïnduceerde c-FOS en p-ERK expressie in de SCN van nacht-actieve knaagdieren. Onze resultaten laten zien dat zowel slaapdeprivatie als cafeïne licht-geïnduceerde c-FOS expressie in de ventrale SCN versterken. Dit wijst er op dat de feedback van gedragsactivatie op cellulaire SCN activiteit en de interactie met de licht-geïnduceerde expressie van zogenaamde "immediate early genes (IEGs)" tegengesteld is in nacht-actieve en dag-actieve dieren. We hebben een deel van deze SCN neuronen kunnen feno-typeren als zijnde calbindin-positief in zowel slaap gedepriveerde als cafeïne behandelde dieren.

Hogere concentraties van serotonine in de SCN en andere hypothalame kernen correleren met circadiane fase-verschuivingen en/of afgenomen licht-geïnduceerde fase-verschuivingen die worden gevonden na slaapdeprivatie in nacht-actieve knaagdieren. Bovendien veroorzaken ook waak-stimulerende farmaca, zoals metamphetamine, een verhoogde afgifte van serotonine in de SCN. Onze resultaten suggereren dat 4 uur slaapdeprivatie de concentratie van serotonine in de SCN en middenhersenen niet veranderd. Cafeïne daarentegen verhoogde de serotonine concentratie in de middenhersenen. Er zijn aanwijzingen voor de aanwezigheid van adenosine receptoren in de SCN. Bovendien induceren micro-injecties van een adenosine agonist in de SCN van hamsters en ratten een fase-verschuiving, net als slaapdeprivatie, en remmen deze stoffen licht-geïnduceerde fase-verschuivingen. Onze data lieten echter geen verschil zien in de hoeveelheid adenosine in de SCN en middenhersenen als gevolg van slaapdeprivatie aan het begin van de nacht of een cafeïne behandeling. Het is dus waarschijnlijk dat in dag-actieve knaagdieren de feedback van gedragsactivatie andere routes gebruikt dan het serotonerge of adenosinerge systeem, misschien het orexine systeem.

Conclusie

In dit proefschrift zijn verschillende aspecten van de interactie tussen slaap deprivatie, circadiane klok en metabolisme in nacht-actieve en dag-actieve soorten behandeld. In het tweede hoofdstuk hebben we verder gekeken naar de SCN controle van het glucosemetabolisme door te onderzoeken hoe centraal GRP het glucosemetabolisme

beïnvloedt. Met behulp van i.c.v. toegediend GRP en de stabiele isotoop verdunningstechniek om endogene glucose productie (EGP) te kunnen bepalen, konden we aantonen dat centrale GRP activiteit resulteert in hyperglycaemie, alsmede een verhoogde EGP en verhoogde plasma glucagon en insuline concentraties. Dit experiment suggereert dat de GRP-geïnduceerde hyperglycaemie het gevolg is van een verhoogde afgifte van glucagon door de pancreas en/of een verhoogde EGP. Vervolg studies zijn nodig om duidelijk te maken of dit inderdaad een SCN-gemedieerd GRP effect is of niet. Glucose homeostase wordt ook beïnvloedt door veranderingen in het slaap/waak patroon, inclusief slaapdeprivatie. In het derde hoofdstuk hebben we in ratten de acute effecten van een korte slaapdeprivatie op glucosetolerantie onderzocht. De resultaten van deze studie laten zien dat onvoldoende slaap resulteert in een verslechtering van de glucosetolerantie, hetgeen beschouwd wordt als een pre-diabetische conditie. Aangezien het ritme in plasma glucose concentratie en de expressie van GRP in de SCN tegengestelde ritmes vertonen in de nacht-actieve muizen en de dag-actieve gras rat, *Arvicanthis ansorgei*, lijkt het interessant om te gaan onderzoeken of de effecten van GRP en slaapdeprivatie op glucose metabolisme ook afhankelijk zijn van de tijd van de dag het dag-actieve of nacht-actieve "karakter" van een soort.

In het vierde hoofdstuk hebben we onderzocht hoe gedragsactivatie het functioneren van de circadiane klok beïnvloedt in een dag-actief knaagdier, *Arvicanthis ansorgei*. Verschillende studies in nacht-actieve knaagdieren hebben reeds laten zien dat gedragsactivatie zorgt voor feedback naar de SCN door het veroorzaken van fase-verschuivingen en het reduceren van photische fase-verschuivingen. In tegenstelling tot wat gevonden is in nacht-actieve knaagdieren vonden wij dat slaapdeprivatie tijdens het begin van de slaapperiode zorgt voor fase-vertraging in ons dag-actieve knaagdier. Gedragsactivatie als gevolg van slaapdeprivatie of cafeïne versterkte het effect van photische fase-verschuivingen, onder andere door het activeren van calbindin-positieve neuronen in de SCN, maar zonder een duidelijke rol voor het serotonine of adenosine systeem. In tegenstelling tot nacht-actieve knaagdieren vertonen dag-actieve knaagdieren geen licht-geïnduceerde slaperigheid, maar vertonen ze juist een toegenomen waakzaamheid als gevolg van licht, net als mensen. Het lijkt daarom belangrijk om ook vragen naar de schadelijke (circadiane) effecten van bijvoorbeeld ploegendienst en jet-lag in dit diermodel te onderzoeken.

RESUME

Les rythmes circadiens chez les mammifères nocturnes et diurnes sont contrôlés par une horloge principale localisée dans le noyau suprachiasmatique (SCN) de l'hypothalamus, horloge principalement mise à l'heure locale par le cycle lumière-obscurité. Le SCN contrôle les rythmes comportementaux et métaboliques en relayant des informations synchronisatrices vers les tissus périphériques. Par exemple, les rythmes journaliers de veille-sommeil, d'alimentation-jeûne, de glycémie, de tolérance au glucose et de sensibilité à l'insuline sont réglés par l'horloge SCN.

Afin de maintenir l'homéostasie du glucose, le SCN transmet des signaux temporels par le système nerveux autonome via des structures-relais hypothalamiques, tels que le noyau hypothalamique paraventriculaire (PVN) et les neurones synthétisant l'orexine dans la zone hypothalamique périfornicale (PF). Des signaux rythmiques excitateurs et inhibiteurs provenant des SCN vers ces structures-cibles (PVN et PF) participent au rythme quotidien glycémie via des afférences sympathiques au foie. D'autres neuropeptides que l'orexine ont également été impliqués dans le contrôle hypothalamique de l'homéostasie du glucose. Plusieurs neuropeptides tels que l'arginine vasopressine (AVP), le peptide intestinal vasoactif (VIP) et la bombésine (Gastrin-releasing peptide, GRP) présentent un rythme quotidien de libération à partir du SCN. Ces neuropeptides pourraient communiquer des signaux temporels aux organes métaboliques via des signaux nerveux et humoraux afin de réguler la rythmicité quotidienne des processus métaboliques. Il est possible que ces neuropeptides soient également impliqués dans le contrôle par le SCN de l'homéostasie du glucose.
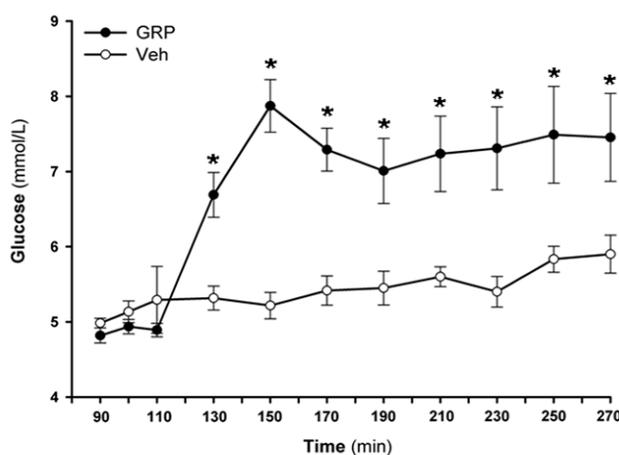
Figure 1. Changements de concentrations en glucose sanguin pendant une infusion icv de GRP. Cette infusion augmente significativement la glycémie. Veh, véhicule. Les données présentées sont des moyennes ± ETM. * $p < 0.05$

Les signaux médiés par le GRP influencent de nombreuses fonctions dans le système nerveux central, y compris la prise alimentaire, la masse corporelle, le métabolisme du glucose et les rythmes circadiens. Dans cette thèse, nous avons étudié l'implication possible du GRP dans le contrôle de l'homéostasie de la glycémie (chapitre 2). Nous avons montré que l'administration intracérébroventriculaire (icv) du GRP provoque rapidement une hyperglycémie qui se maintient jusqu'à la fin de l'infusion (fig. 1). L'augmentation des niveaux de glucose plasmatique s'accompagne d'augmentation de la production endogène de glucose (EGP), ainsi qu'une élévation des concentrations plasmatiques de glucagon et d'insuline (Fig. 2 A, B et C). En outre, aucune différence dans les niveaux plasmatiques de corticostérone n'est observée entre les rats témoins et ceux traités au GRP (Fig. 2 D).
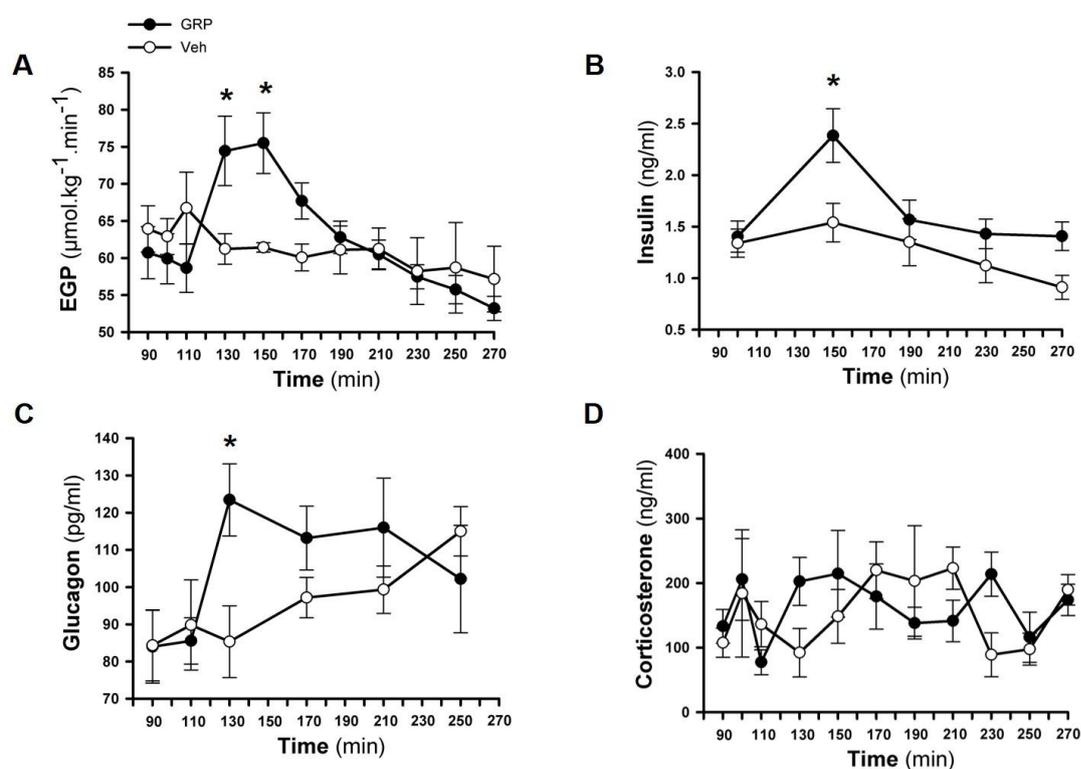

Figure 2. Variations de production endogène de glucose (EGP) et hormones glucorégulatrices pendant l'infusion icv de GRP. Ce traitement augmente significativement l'EGP (A), l'insulinémie (B) et la glucagonémie (C). La corticostéronémie reste inchangée (D). Veh, véhicule. Les données présentées sont des moyennes ± ETM. * $p < 0.05$

L'excitation et l'inhibition rythmique des neurones pré-autonomes contrôlées par le SCN dans le PVN contribuent à maintenir l'homéostasie journalière du glucose via les afférences du système nerveux autonome vers le foie. Le contenu en GRP du SCN chez le rat augmente pendant la journée et diminue progressivement au cours de la nuit. De plus, le récepteur GRP (GRP-R) et sa capacité de liaison varient au cours du cycle avec une acrophase en début de

nuit, comme le rythme de glucose plasmatique. Mais on ne sait pas encore si l'expression du GRP-R dans le PVN est rythmique ou non. L'administration icv de GRP augmente l'expression de c-FOS dans les SCN et PVN. Cependant, on ne sait pas quelles cellules des SCN et PVN sont spécifiquement activées par le traitement au GRP. Sur la base de ces résultats, nous émettons l'hypothèse que l'hyperglycémie induite par le GRP icv pourrait impliquer une activation des neurones du PVN par le GRP, ce qui entraînerait une activation sympathique du pancréas et du foie, conduisant ainsi à la suite une libération accrue de glucagon et à une EGP augmentée.

Les concentrations plasmatiques de glucose suivent un rythme quotidien qui est en antiphase entre rongeurs diurnes et nocturnes. Il n'y a encore aucun argument expérimental qui indique que les neuropeptides du SCN varient en opposition de phase entre les espèces nocturnes et diurnes. Il faut néanmoins noter que les profils journaliers de libération de la plupart des neuropeptides des SCN ne sont pas connus en détail chez les rongeurs nocturnes et qu'ils n'ont quasiment pas été étudiés du tout chez les animaux diurnes. Afin de mieux comprendre la régulation de l'homéostasie du glucose par le GRP chez les espèces nocturnes et diurnes, nous aurons besoin d'effectuer des expériences de chronopharmacologie au cours du cycle journalier aussi bien chez les rats nocturnes que chez des rongeurs nocturnes diurnes, tels que le Rat roussard du Soudan, *Arvicanthis ansorgei*.

Le SCN contrôle non seulement le rythme journalier de glucose plasmatique, mais il régule également le rythme d'utilisation du glucose. Tant dans les études humaines qu'animales, la tolérance au glucose est plus élevée au début de la période journalière d'activité, à savoir : le matin chez les humains et le soir chez les rongeurs nocturnes. L'essor récent de données provenant d'études épidémiologiques et expérimentales révèle que la réduction de la durée du sommeil augmente les risques métaboliques, à savoir : obésité, diabète de type 2 et problèmes cardiovasculaires. Des études antérieures chez des sujets ont montré que la réduction du temps de sommeil ou une mauvaise qualité de sommeil altère la tolérance au glucose. Dans notre étude, nous avons étudié l'effet d'une restriction aiguë de sommeil sur la tolérance au glucose chez les rats (chapitre 3). Un groupe d'animaux a été privé de sommeil pendant 4 h au début de la période de repos et un autre groupe a été privé de sommeil durant le milieu de la période de repos. Immédiatement après la période de privation de sommeil, nous avons effectué des tests de tolérance au glucose par voie intraveineuse (IVGTT). Les résultats de cette étude démontrent que seulement 4 h de privation de sommeil altère sévèrement la tolérance au glucose évaluée par IVGTT et réduit en particulier la réponse précoce de l'insuline après une surcharge en glucose et ce, indépendamment de la fenêtre temporelle, à

savoir, en début ou au milieu de la période de repos (Fig. 3 A , B, D, E). Il est intéressant de noter que les concentrations basales en glucose et en insuline sont restées inchangées avant et après la privation de sommeil. Nous avons également constaté que les concentrations plasmatiques de corticostérone ne différaient pas entre le groupe témoin et celui privé de sommeil, ce qui exclut une éventuelle activation de l'axe corticotrope pour expliquer la détérioration de la tolérance au glucose pendant l'IVGTT (Fig. 3 C, F). Ces résultats suggèrent que, indépendamment de l'heure du jour et de la pression du sommeil, une privation de sommeil aiguë et de courte durée induit une intolérance au glucose chez le rat en atténuant la réponse précoce d'insuline à une surcharge en glucose.
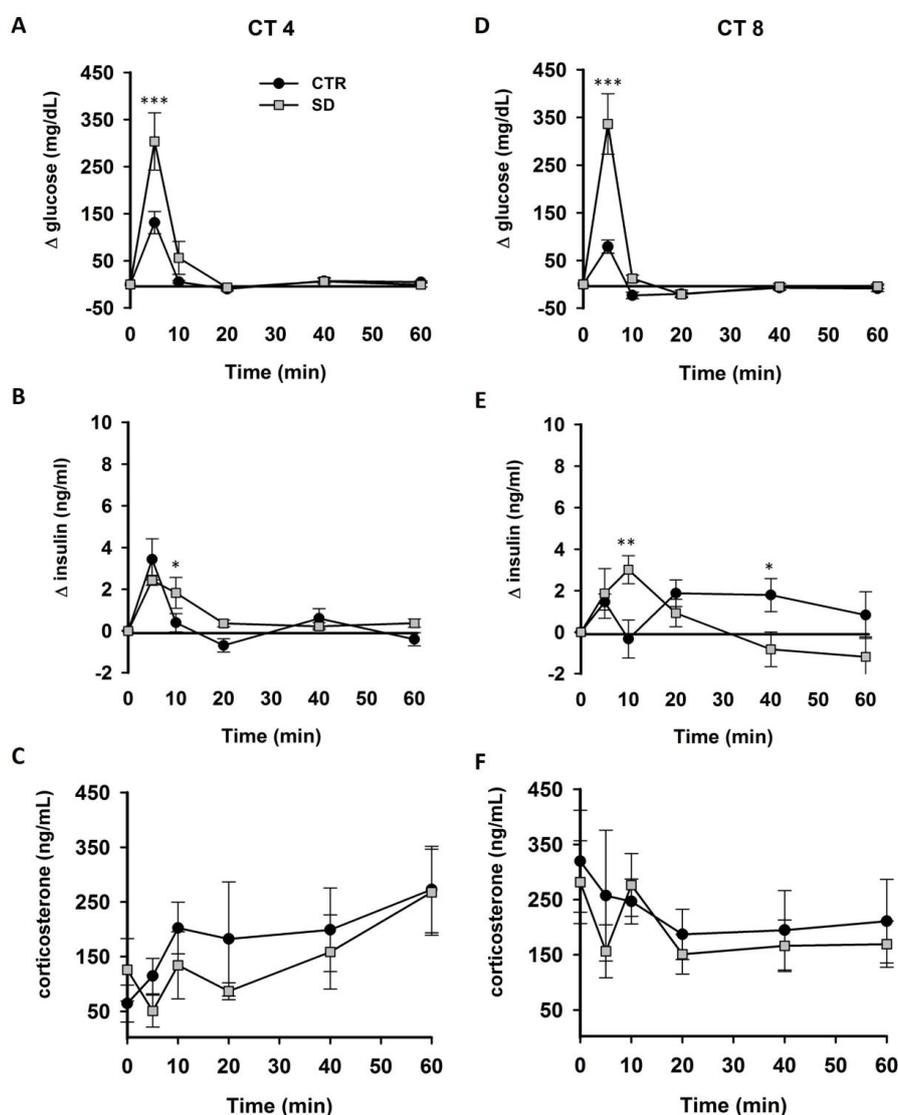

Figure 3. Tests de tolérance au glucose par voie intraveineuse (IVGTT) chez des rats privés de sommeil pendant 4 h d'affilée. Changements relatives de glycémie (**A**, **D**), changements relatifs d'insulinémie (**B**, **E**) et de corticosteronémie (**C**, **F**) après une injection de glucose (500 mg·kg^{-1} par voie intraveineuse) pendant un IVGTT démarré au temps circadien (CT) 4 ou CT8. Cercles noirs: animaux témoins; carrés gris: animaux privés de sommeil. CTR = groupe contrôle, SD = groupe privé

de sommeil. n = 6 par groupe. Les données présentées sont des moyennes ± ETM. * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$

L'élimination du glucose plus lente chez les animaux privés de sommeil pendant l'IVGTT peut résulter d'une augmentation de production de glucose ou d'une absorption réduite. Les données dont nous disposons ne nous permettent pas de déterminer avec précision la cause de l'hyperglycémie observée. La diminution de la réponse insulinique précoce dans le groupe privé de sommeil peut aussi bien conduire à une réduction de l'absorption du glucose ou à une désinhibition de la production de glucose. Des expériences utilisant la technique de dilution des isotopes stables pour déterminer la production de glucose endogène (EGP) seront nécessaires pour comprendre la nature de l'hyperglycémie observée chez les animaux privés de sommeil pendant IVGTT. Des travaux antérieurs ont montré que la privation de sommeil active la branche sympathique du système nerveux autonome et provoque la libération de catécholamines dans la circulation générale. La réduction de la réponse précoce de l'insuline à une surcharge en glucose pourrait être associée à une activation du système sympathique et/ou là une réduction de l'activité parasympathique. Maintenant, la question qui se pose est la suivante: quelles pourraient être les structures centrales qui contrôlent les effets périphériques observés lors de la privation de sommeil ? Nous émettons l'hypothèse que le candidat probable est le système orexinergique hypothalamique, en raison de son implication dans la régulation du cycle veille sommeil et du métabolisme du glucose. Un autre candidat putatif est le système du neuropeptide Y (NPY) hypothalamique en raison de son implication dans la régulation de l'éveil et du métabolisme du glucose.

De nouvelles expériences vont être nécessaires pour déterminer si l'effet de la privation de sommeil sur le métabolisme du glucose implique l'orexine et/ou le NPY. De plus, le cycle veille-sommeil est opposé entre les espèces nocturnes et diurnes en fonction du cycle astronomique de lumière et d'obscurité, tandis que la glycémie présente également des variations journalières en opposition de phase entre rongeurs nocturnes et diurnes. Par conséquent, il serait intéressant d'effectuer des expériences similaires chez les rongeurs et diurnes pour étudier si la privation de sommeil altère le métabolisme du glucose et dans quelles mesures. Disséquer les mécanismes qui conduisent à une altération de l'homéostasie du glucose en réponse à une restriction de sommeil est clairement pertinent pour les applications biomédicales.

La privation de sommeil et d'autres procédures d'éveil comportemental partagent la propriété de pouvoir moduler la phase et la période de l'horloge principale dans le SCN, impliquant des

signaux provenant d'autres zones du cerveau que le SCN. Chez les rongeurs nocturnes, l'éveil comportemental induit des déphasages circadiens et réduit les déphasages du rythme d'activité locomotrice induite par la lumière. Chez ces animaux, l'activation des voies sérotonergiques provoque des déphasages du SCN pendant le jour et atténue les décalages de phase induits la lumière de nuit. Au contraire, chez les animaux diurnes, l'activation des voies sérotonergiques produit des déphasages de nuit et améliore les décalages de phase induits par la lumière. Ainsi, le rôle du système de rétroaction sérotonergique semble agir de manière opposée entre les animaux diurnes et nocturnes. La privation de sommeil chez les rongeurs nocturnes peut décaler l'horloge SCN pendant le jour et diminuer les déphasages induits par la lumière de nuit. Chez les rongeurs diurnes, cependant, le rôle de l'éveil comportemental ou de la privation de sommeil dans ces fonctions n'a pas encore été étudié de manière approfondie. Cela nous a incité à étudier plus en détail, chez les animaux diurnes, la rétroaction possible de l'éveil comportemental sur l'horloge du SCN horloge et les déphasages induits par la lumière. Nous avons commencé par tester l'effet de la privation de sommeil puis celui de l'éveil induit par la caféine sur le fonctionnement de l'horloge SCN et sa sensibilité à la lumière chez un rongeur diurne, le Rat roussard du Soudan, *Arvicanthis ansorgei* (chapitre 4). Les déphasages de l'activité locomotrice ont été analysés chez des rats roussards transférés d'un cycle lumière-obscurité en obscurité constante et testés en début de nuit (privation de sommeil de 4 h, du temps circadien 12 (CT12) à CT16, ou injection de caféine à CT14) et plus tard dans la nuit (privation de sommeil de CT16 à CT20, ou injection de caféine à CT18) et les animaux ont été ou non exposés à une stimulation lumineuse de 30 minutes à CT16 et CT20, respectivement.
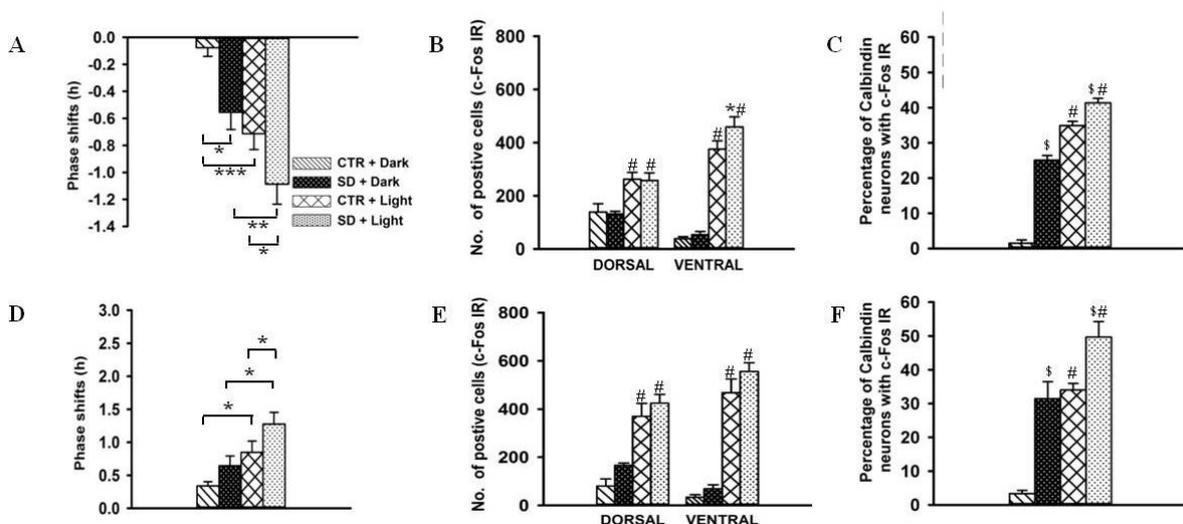

Figure 4. La privation de sommeil potentialise les déphasages induits par la lumière (A) En début de nuit, la privation de sommeil induit des retards de phase. Les retards de phase induits par la lumière à

CT 16 sont potentialisés par la privation de sommeil. (B) En début de nuit, la privation de sommeil augmente l'expression de c-FOS induite par la lumière dans le SCN ventral. (C) En début de nuit, la privation de sommeil, avec ou sans exposition à la lumière, induit l'expression de c-FOS dans les neurones à calbindine. (D) Les avances de phase induites par la lumière à CT 20 sont potentialisées par la privation de sommeil. (E) En fin de nuit, la privation de sommeil n'a pas d'effet significatif sur l'expression de c-FOS induite par la lumière dans le SCN ventral. (F) En fin de nuit, la privation de sommeil, avec ou sans exposition à la lumière, induit l'expression de c-FOS dans les neurones à calbindine. * $P < 0,05$, ** $p < 0,005$, *** $p < 0,001$, # $p < 0,001$ entre les conditions de lumière et d'obscurité, \$ $p < 0,001$ entre les groupes privés de sommeil et contrôles. SD, privation de sommeil ; CTR, contrôle ; CAF, caféine ; VEH, véhicule.

L'analyse des décalages de phase montre que la privation de sommeil induit des déphasages importants (retards) et potentialise les retards et les avances de phase induits par la lumière en début et fin de nuit (Fig. 4 A, D). La privation de sommeil en début de nuit augmente l'expression de c-FOS induite par la lumière dans le SCN ventral (Fig. 4 B, E). Nous avons phénotypé ces neurones en sélectionnant la calbindine comme candidat putatif. La privation de sommeil en début et fin de nuit a augmenté spécifiquement l'expression de c-FOS dans les cellules SCN contenant la calbindine, indiquant que ces cellules sont activées (Fig. 4 C, F). En outre, alors que la caféine seule n'a pas d'effet de déphasage en début et fin de nuit, ce traitement augmente les déphasages, à la fois les retards et les avances de phase, induits par la lumière (Fig. 5 A, D). En ce qui concerne le marquage c-FOS, il est augmenté par la caféine seule en fin de nuit dans la région dorsale des SCN. En outre, l'induction de c-FOS dans le SCN ventral par la lumière est augmentée à la fois en début et en fin de nuit par la caféine (Fig. 5 B, E). Ici encore, nous avons phénotypé ces neurones en sélectionnant la calbindine comme candidat. Le traitement à la caféine en début et fin la nuit a augmenté l'expression de c-FOS dans les cellules immunoréactives à la calbindine des SCN (Fig. 5 C, F).
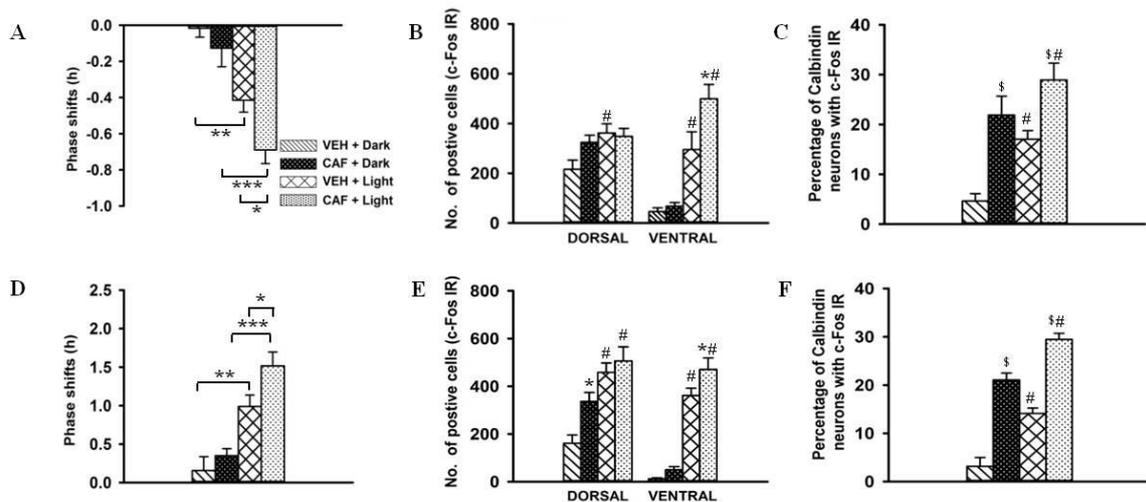

Figure 5. La caféine potentialise les déphasages provoqués par la lumière. (A) Les retards de phase induits par la lumière à CT 16 sont potentialisés par le traitement à la caféine. (B) L'injection de de caféine à CT 14 augmente l'expression de c-FOS induite par la lumière dans le SCN ventral. (C) Le

traitement à la caféine à CT 14 induit l'expression de c-FOS les cellules à calbindine, avec ou sans lumière. (D) Les avances de phase induites par la lumière à CT 20 sont potentialisées par le traitement à la caféine. (E) La caféine à CT 18 induit l'expression de c-FOS dans le SCN dorsal et augmente l'induction de c-FOS par la lumière dans le SCN ventral. (F) Le traitement à la caféine à CT 18 active l'expression de c- dans les neurones à calbindine, avec ou sans lumière. * P <0,05, ** p <0,005, *** p <0,001, # p <0,001 entre conditions de lumière et d'obscurité, $ p <0,001 entre les groupes privés de sommeil et les contrôles. SD, privation de sommeil ; CTR, contrôle ; CAF, caféine ; VEH, véhicule.

Nous avons également évalué les niveaux de sérotonine (5-HT) et d'adénosine dans le SCN et le mésencéphale après privation de sommeil ou traitement à la caféine en début de nuit subjective (CT14). Les niveaux de 5-HT ne sont pas différents dans le SCN et le mésencéphale après privation de sommeil. Cependant, le traitement à la caféine provoque une augmentation des concentrations de 5-HT dans le mésencéphale (Fig. 6 A, B). Les concentrations d'adénosine dans le mésencéphale et SCN n'ont pas été modifiées après la privation de sommeil ou le traitement à la caféine (Fig. 6 C, D).
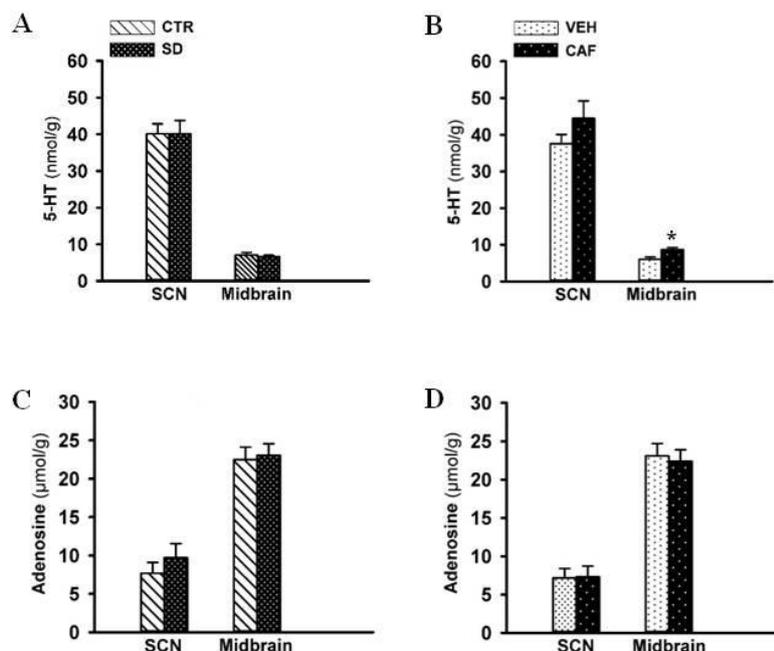

Figure 6. Concentrations dans le SCN et le mésencéphale de sérotonine (5-HT, graphiques A, B) et d'adénosine (graphiques C, D) après une privation de sommeil en début de nuit (CT12-16) ou après injections de caféine/véhicule à CT14 chez le rongeur diurne, *Arvicanthis*. * P <0,01 entre groupe caféine et groupe véhicule; SD, privation de sommeil ; CTR, contrôle ; CAF, caféine ; VEH, véhicule.

Des études précédentes chez les rongeurs nocturnes ont montré que la privation de sommeil ou l'activité locomotrice forcée en milieu de période de repos induit des avances de phase. Une injection d'agoniste des récepteurs A1 de l'adénosine en milieu de période de repos, une procédure visant à imiter les effets de la privation de sommeil par voie pharmacologique, induit également une avance de phase d'une manière dose-dépendante. Plusieurs séries

d'expériences ont conduit au schéma général que les courbes de réponse de phase (PRC) à l'éveil comportemental sont de type « non-photique », avec des avances de phase maximales pendant le milieu de la période habituelle de repos (jour subjectif) et parfois des retards de phase au cours de la seconde moitié de la période d'activité habituelle (nuit subjective). Dans cette étude, nous montrons que la privation de sommeil en début de période de repos (nuit) chez le rongeur diurne, *Arvicanthis ansorgei,* induit des retards de phase, alors que la privation de sommeil en fin de nuit n'a provoqué aucun changements significatif de phase. En accord avec les études précédentes menées chez les souris et les hamsters nocturnes, la caféine seule n'a pas provoqué de déphasage. Ces résultats indiquent que les périodes circadiennes de sensibilité à l'éveil comportemental dépendent du cycle activité-repos qui est opposé entre espèces nocturnes et diurnes.

Les facteurs d'éveil interagissent avec l'entraînement photique. Par exemple, chez les rongeurs nocturnes, la privation aiguë de sommeil, l'activité forcée, ou des injections d'un agoniste des récepteurs A1 de l'adénosine réduisent les déphasages induits par la lumière. De manière inattendue, la caféine, qui est un antagoniste de l'adénosine, bloque également les retards de phase induits par la lumière chez les souris, comme d'autres molécules qui stimulent la vigilance telles que la méthamphétamine et le modafinil. Nos données montrent que la privation de sommeil et le traitement à la caféine potentialisent les déphasages (avances et retards) induits par la lumière chez un rongeur diurne. Ces résultats suggèrent que l'éveil comportemental module l'entraînement photique de l'horloge circadienne de manière opposée entre rongeurs nocturnes et diurnes.

L'induction de c-FOS par la lumière dans le SCN, qui est similaire entre les rongeurs nocturnes et diurnes, a été utilisée comme marqueur fonctionnel. Les travaux antérieurs effectués chez les rongeurs nocturnes ont montré que l'éveil comportemental réduit l'expression c-FOS dans le SCN et il réduit aussi l'induction de c-FOS et p-ERK par la lumière dans le SCN. Nos résultats montrent que les deux procédures expérimentales que nous avons utilisées pour stimuler l'éveil, à savoir : privation de sommeil et caféine, augmentent l'expression induite par la lumière de c-FOS dans le SCN ventral. Ainsi, les effets de rétroaction de l'éveil comportemental sur le SCN et sa réponse moléculaire à la lumière (activation de gènes précoces, en l'occurrence) sont opposés entre espèces nocturnes et diurnes. Nous avons également mis en évidence que les cellules activées par la privation de sommeil et la caféine contenaient la calbindine.

Des concentrations accrues de la sérotonine dans le SCN et d'autres régions hypothalamiques après la privation de sommeil ont été corrélées avec les déphasages circadiens et/ou la

réduction des déphasages induits par la lumière chez les rongeurs nocturnes. En outre, les produits pharmaceutiques qui promeuvent l'éveil, comme la méthamphétamine, augmentent également la libération de sérotonine dans le SCN. Nos résultats suggèrent qu'une privation de sommeil pendant 4 h n'a pas modifié la concentration tissulaire en sérotonine dans le SCN et le mésencéphale. En revanche, l'injection de caféine augmente la concentration en sérotonine dans le mésencéphale. Les récepteurs de l'adénosine sont exprimés dans le SCN. De plus, des microinjections d'agoniste adénosinergique dans le SCN des hamsters et des rats induisent des déphasages circadiens, tout comme la privation de sommeil, et ces composés atténuent également les déphasages induits par la lumière. Cependant, nous n'avons pas détecté de différence de concentration en adénosine dans le SCN et le mésencéphale après privation de sommeil ou injection de caféine en début de nuit. Par conséquent, il semble que la rétroaction de l'éveil comportemental chez les rongeurs diurnes suive d'autres voies que les sérotonergiques et adénosinergiques (par exemple, orexinergiques).

Conclusion

La thèse présente traite de plusieurs aspects des interactions entre privation de sommeil, horloge circadienne et métabolisme chez les espèces nocturnes et diurnes. Dans le deuxième chapitre de cette thèse, nous avons approfondi l'investigation sur le contrôle par le SCN du métabolisme du glucose en étudiant l'action centrale du GRP sur l'homéostasie du glucose. En utilisant des perfusions icv de GRP et la technique de dilution des isotopes stables pour déterminer la production de glucose endogène (EGP), nous montrons que l'action centrale du GRP conduit à une hyperglycémie accompagnée d'une augmentation de la EGP, ainsi qu'une augmentation des concentrations plasmatiques de glucagon et d'insuline. Par conséquent, cette expérience démontre que l'hyperglycémie induite par le GRP est probablement due à la libération glucagon pancréatique et/ou à la EGP. D'autres études seront nécessaires pour comprendre si cet effet est médié ou non par le SCN. L'homéostasie du glucose est également affectée par des altérations du cycle veille/sommeil, y compris la privation de sommeil. Dans le troisième chapitre, nous avons étudié les effets de la privation de sommeil aiguë à court terme sur la tolérance au glucose chez les rats. Nos résultats indiquent que le manque de sommeil conduit à une altération de la tolérance au glucose, qui est considérée comme une condition pré-diabétique. Puisque la glycémie et l'expression de GRP dans le SCN montrent des rythmes anti-phasiques entre les rongeurs nocturnes et diurnes, il sera intéressant d'étudier si les effets du GRP et de la privation de sommeil sur le métabolisme du glucose dépendent de l'heure du jour et du caractère nocturne diurne de l'espèce étudiée.
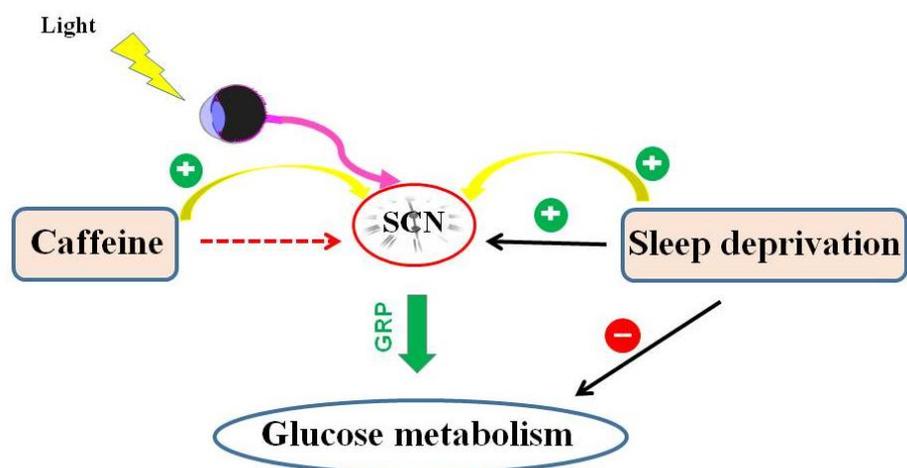
Figure 7. Effets de la privation de sommeil et du traitement à la caféine sur l'horloge circadienne du SCN et le métabolisme du glucose

Dans le quatrième chapitre, nous avons cherché à savoir comment l'éveil comportemental affecte le fonctionnement de l'horloge circadienne chez le rongeur diurne, *Arvicanthis ansorgei*. Diverses études chez les rongeurs nocturnes ont démontré que l'éveil comportemental agit sur le SCN en déphasant l'horloge principale pendant le jour et en réduisant sa mise à l'heure par la lumière. Contrairement à ce qui est observé chez les rongeurs nocturnes, nous montrons que la privation de sommeil en début de période de repos induit des retards de phase chez ce rongeur diurne. De plus, l'éveil provoqué soit par la privation de sommeil ou le traitement à la caféine potentialise les déphasages induits par la lumière, en partie par l'activation des cellules suprachiasmatiques contenant la calbindine et sans impliquer les systèmes sérotoninergique et adénosinergique. Contrairement aux rongeurs nocturnes, les rongeurs diurnes ne présentent pas de photosomnolence. A la place, ils répondent par une vigilance accrue à l'exposition à une lumière vive, comme les humains. Par conséquent, il sera important d'approfondir la question de la désynchronisation circadienne, y compris le travail posté et les voyages trans-méridiens chez ce modèle de rongeur diurne, et de trouver les moyens d'y remédier.

PhD Portfolio

Pawan Kumar Jha
PhD period: October 2012 – May 2016
Name of PhD supervisors: Dr. Etienne Challet and Prof. Dr. Andries Kalsbeek

Courses

-Laboratory animal science	2012
-Neurex workshop on sleep and circadian rhythm	2013
-Basic course in Neuroendocrinology (ESE)	2014
-Clinic, genetics and pathophysiology of neurodegenerative disease	2014

Diploma obtained

-Article 9 (Certification for animal experiments in the Netherlands)	2012

Conferences attended

-CTR Meeting, Netherland Institute of Neuroscience, Amsterdam, Netherlands	2012
-1st NeuroTime Annual Meeting, Beuggen, Germany *(Oral presentation)*	2013
-Biennial Colloquium, Nutrition Metabolism and the Brain, Amsterdam, Netherlands	2013
-XIII European Biological Rhythm Society (EBRS) congress, Munich, Germany. (*Poster presentation*)	2013
-Neurex meeting on understanding the neural basis of diurnality, Strasbourg, France	2013
-European society of Endocrinology conference, Amsterdam, Netherlands (*Poster presentation*)	2014
-2nd NeuroTime Annual Meeting, Amsterdam, Netherlands *(Oral presentation)*	2014
-3rd NeuroTime Annual Meeting, Basel, Switzerlands *(Oral presentation)*	2015
-XIV European Biological Rhythm Society (EBRS) congress, Manchester, England. (*Oral presentation*)	2015
-International Associated Laboratory LIA1061 CNRS, Strasbourg, France. *(Oral presentation)*	2015
-4th NeuroTime Annual Meeting, Strasbourg, France *(Oral presentation)*	2015

Publications

1. Pawan Kumar Jha, Ewout Foppen, Etienne Challet and Andries Kalsbeek, Effects of central-gastrin releasing peptide on glucose metabolism, *Brain Research*, **1625**, 135-141 (**2015**).

2. Pawan Kumar Jha, Etienne Challet and Andries Kalsbeek, Circadian rhythms in glucose and lipid metabolism in nocturnal and diurnal mammals, *Molecular Cellular Endocrinology*, **418**, 74-88 (**2015**).

3. Pawan Kumar Jha, Ewout Foppen, Andries Kalsbeek and Etienne Challet, Sleep restriction acutely impairs glucose tolerance in rats *Accepted in Physiological Reports.*

4. Pawan Kumar Jha, Hanan Bouaouda, Sylviane Gourmelen, Stephanie Dumont, Yannick Goumon, Andries Kalsbeek and Etienne Challet, Sleep deprivation and caffeine treatment potentiate photic resetting of circadian clock in a diurnal rodent, the Sudanian grass rat (*Arvicanthis ansorgei*) *Ready for submission*.

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PAWAN KUMAR JHA

SLEEP DEPPRIVATION AND ITS IMPACT ON CIRCADIAN RHYTHMS AND GLUCOSE METABOLISM

Résumé

Situé dans le noyau suprachiasmatique (SCN) de l'hypothalamus, l'horloge principale contrôle les rythmes des processus comportementaux et métaboliques chez les mammifères. Par exemple, les rythmes quotidiens de veille-sommeil, d'alimentation-jeûne, de glycémie, de tolérance au glucose et de sensibilité à l'insuline sont régulés par l'horloge SCN. La lumière est le synchroniseur principal du SCN, même si de nombreux facteurs autres que la lumière, tels que l'éveil comportemental ou des facteurs métaboliques, peuvent également moduler la phase ou la période des SCN. L'objectif de cette thèse était d'étudier différents aspects des interactions entre l'éveil comportemental, les rythmes circadiens et le métabolisme du glucose. Dans la première partie, nous avons déterminé l'action centrale du *Gastrin-Releasing Peptide* (GRP), un neuropeptide synthétisé dans le SCN, sur le métabolisme du glucose. Nos résultats indiquent qu'une injection icv de GRP induit une hyperglycémie prolongée. Nous avons également montré qu'une privation de sommeil à court terme conduit à une détérioration de la tolérance au glucose. Dans la deuxième partie, nous avons démontré que l'éveil comportemental induit par la privation de sommeil ou une injection de caféine améliore l'entraînement photique de l'horloge SCN chez un rongeur diurne : le rat roussard du Soudan, *Arvicanthis ansorgei*. Ces réponses circadiennes chez une espèce diurne qui sont opposées à celles précédemment mises en évidence chez les rongeurs nocturnes pourraient avoir des applications biomédicales.

Mots-clefs : Eveil comportemental, rythmes circadiens, déphasages, métabolisme du glucose, diabète de

Abstract

Located in the hypothalamic suprachiasmatic nucleus (SCN), the master clock generates rhythms of behavioural and metabolic processes in mammals. For example, daily rhythms of sleep-wake, fasting-feeding, plasma glucose concentration, glucose tolerance and insulin sensitivity are regulated by the SCN clock. Light is the primary synchronizer of SCN pacemaker though many light-independent factors such as behavioural arousal and metabolic cues also have phase and period resetting properties. The aim of thesis was to study different aspects of the interactions between behavioural arousal, circadian rhythms and glucose metabolism. In the first part, we extended the study of brain control of glucose metabolism by investigating the central action of gastrin-releasing peptide (GRP), a neuropeptide synthesized in the SCN, on glucose metabolism. Our result indicates that central GRP induces long-lasting hyperglycemia. We also showed that acute sleep deprivation leads to impaired glucose tolerance. In the second part, we demonstrated that behavioural arousal induced by sleep deprivation or caffeine treatment enhances photic-entrainment of the SCN clock in the diurnal Sudanian grass rat, *Arvicanthis ansorgei*. These circadian responses in a diurnal species are opposite to the earlier findings in nocturnal rodents and may have biomedical applications.

Key words: Behavioural arousal, Circadian rhythms, Phase-shifts, Glucose metabolism, Type 2 diabetes