THE SUPRACHIASMATIC NUCLEUS ORGANIZES DAILY PHYSIOLOGY

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An analysis of the role of the autonomic nervous system and peripheral clock genes

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A mes parents

Anubis, by Eric Cailotto

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Pour un esprit scientifique, toute connaissance est une réponse à une question.
S'il n'y a pas eu de question, il ne peut y avoir connaissance scientifique.
Rien ne va de soi. Rien n'est donné. Tout est construit.

Extrait de La Formation Scientifique, Gaston Bachelard. Paris, Vrin, 7e ed., 1970, p.14.

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SCOPE OF THE THESIS

HOMEOSTASIS AND ANTICIPATION TO CHANGES

Homeostasis

The study of internal processes and functions that ensure and maintain the life of the organism was called 'physiology' for the first time by Jean Francois Fernel in 1554. Darwin (1809-1882) demonstrated the considerable influence of the environment on the living organism. The living creature is subjected to various external fluctuations (i.e., food availability, temperature, predation, etc.) and the organism therefore has to adapt to its environment in order, not only to survive, but to maintain its own species. At the same time, Claude Bernard (1813-1877) proposed another notion, i.e. the *interior milieu,* which is formed by an organic fluid surrounding the organs of the individual. Bernard suggested that maintaining the stability of the internal fluid (blood and other body fluids) is essential for the life of higher organisms, as multiple and/or chronic disturbances of this *interior milieu* would lead to pathology. Afterwards, Walter B. Cannon (1871-1945) defined the notion of "*fixité du milieu intérieur*" more precisely with the term Homeostasis (*Homeo* = same; *stasis* = steadyness). Homeostasis is the maintenance of equilibrium in the internal system by means of an automatic mechanism that counteracts influences tending towards disequilibrium. This constant set point of the internal fluid can be disrupted under stressful situations or by "emotional" stimulation. Later, the concept of Homeostasis was refined by Donald C. Jackson (1987): "Homeostasis is not a single optimal control condition but rather a variety or continuum that varies with the animal's circumstances. Set points or regulated values are not fixed, but may change depending on ambient conditions or because of changing physiological conditions or demands".

Currently we consider the daily or seasonal changes in the environment as important variables that will influence the homeostatic settings of the organism in order to allow it to anticipate these environmental changes. Especially the structures and mechanisms involved in the anticipation of these daily changes are the subject of this thesis.

Anticipation

Being able to anticipate daily or seasonal changes in the environment will decrease the moments in which homeostasis can be disturbed, and so anticipation will decrease the risk of disease or pathology. Migration was the first periodic/anticipatory behaviour that was studied extensively. A review written by Angus M. Woodbury in 1941 indicated that "migration may be considered as periodic passing of animals from one place to another in which this transition is timed to fit with some periodic rhythm or cycle of the environment". Herewith the notion of biological rhythm came up as periodic changes in physiology and behaviour among species in order to accommodate environmental changes. To adapt to the periodic environmental changes, timed

reproduction is another strategy adopted by the species in order to increase their offspring's chance to survive, i.e. delivery occurs at the period when the environment is conducive to the development of the young, by the availability of food and moderate temperatures [1-4].

In the shorter term, also physiological anticipation to daily changes can be observed. For instance, the "dawn-phenomenon" as observed in humans, whose physiology anticipates the glucose demands for the upcoming activity period, i.e. increased glucose output and insulin sensitivity in the early morning [5].

This rhythmic or periodic/anticipatory property, even on a yearly basis, relies on the capacity of the organism to generate an internal "notion of time" given by a biological clock. In this respect it is interesting that it was recently shown that even butterflies rely on biological clock mechanisms for their orientation for migration

CIRCADIAN CLOCK IN MAMMALS

1. The suprachiasmatic nucleus: the biological clock or master clock

Localization and properties

By performing lesion studies, Richter (1967) was the first to indicate that the medial part of the hypothalamus is involved in the rhythmicity of locomotor activity. Bilateral lesions or ablation of the suprachiasmatic nucleus (SCN) later showed that the integrity of these nuclei is essential for the rhythmicity of drinking and food intake behaviour, locomotor activity, and the sleep-wakefulness cycle but also for hormonal rhythms, such as corticosterone and melatonin [6-10]. Electrical recording showed that the SCN is an endogenous pacemaker, because SCN neurons display circadian pattern in their neuronal firing with high activity during the subjective daytime even when isolated *in vivo* [11] or maintained as a tissue culture *in vitro* [12, 13]. Another approach, using ^{14}C labelled deoxyglucose, corroborated that the clock is an endogenous oscillator by demonstrating a rhythmic pattern in glucose metabolism in the SCN which also persists in constant darkness [14, 15]. The final evidence that the SCN is "the master oscillator" came from studies in which SCN transplants were able to restore circadian behaviour in SCN lesioned animals who would take on the period of the donor graft [16]. The locomotor rhythm was transmitted even after the SCN was encapsulated, indicating that the clock is able to drive locomotor rhythmicity by means of a diffusible signal [16, 17]. However, SCN grafts did not generate neuroendocrine rhythms, indicating that both neuronal and humoral communications are required to spread the complete clock signal [18].

The clockwork

Although autonomous oscillation in isolated single SCN cells has been shown [13], the

intrinsic mechanism which underlies the oscillation in the firing rate of SCN neurons is still not fully understood. Approaches using combined gene targeting and locomotor activity in mice have provided the main knowledge on the mammalian clockwork, i.e. a molecular mechanism underlying the oscillatory properties of the SCN cell.

A first gene, called *Clock*, was found as an essential component for maintaining the rhythmicity in wheel-running activity in mice without light cues [19, 20]. However, recent findings demonstrate that Clock-deficient mice still display a robust circadian pattern of their locomotor activity, which leads to reconsider the role of the clock in the functionality of the SCN clockwork [21]. Three period genes (*Per1, 2 and 3*) were reported in mice also as elements of the clock machinery. Besides the overlapping of their mRNA rhythmic profiles, the authors proposed an interaction between the PERIOD proteins as a part of the machinery process responsible for the rhythmic pattern of their transcript levels. Another clock member family, Cryptochrome 1 and 2 (*Cry1 and 2*) was also found to be essential for maintaining the rhythmic locomotor activity in constant darkness [22]. Later on, PER and CRY were proposed to interact with each other to form a heterodimer, which in turn will inhibit their own transcription, i.e. the negative limb of the clock feedback loop [23]. All the evidence accumulated from mice studies lead to a hypothetical clock mechanism based on interacting positive and negative transcriptional-/translational-feedback loops in which *Bmal1* and *Clock* are part of the positive limb, and *Cry1/2* and *Per1/2/3* are part of the negative limb (Figure 1) [24, 25]. This hypothetical clockwork scheme, which represents the backbone of the molecular circadian clock, is constantly updated by the discovery of new proteins that play a role in the adjustment of the circadian timing system. Of these proteins, Casein Kinase I epsilon is a protein which phosphorylates PER protein and may affect its stability, cellular location and even its interaction with other proteins [26, 27]. REV-ERBα and RORα , two orphan nuclear receptors play an important role in the transcription of the clock gene Bmal1, by repressing or activating its activity, respectively [28-31]. In 2002, Honma and coworkers described another clock-gene family, Dec1 and Dec2, as negative components of the core loop [32, 33]. Despite the fact that the integrity of the clockwork is required to generate circadian rhythms, the question "how this molecular mechanism regulates the electrical activity of SCN neurons and rhythmic biological processes like endocrine or behavioural rhythms" remains open.

Molecular studies have provided a model for the mechanism by which the clockwork is able to control/influence in a circadian manner the transcription of genes through the activation of E-box enhancer (CCG sequence) by CLOCK-BMAL1 heterodimer (two clock components of the positive feedback loop). For instance, the rhythmic transcription of the arginine vasopressin gene (AVP), a neurotransmitter used by the SCN to spread its timing signal, contains an E-box in its promoter region and is thus directly controlled and generated by the clockwork [34].

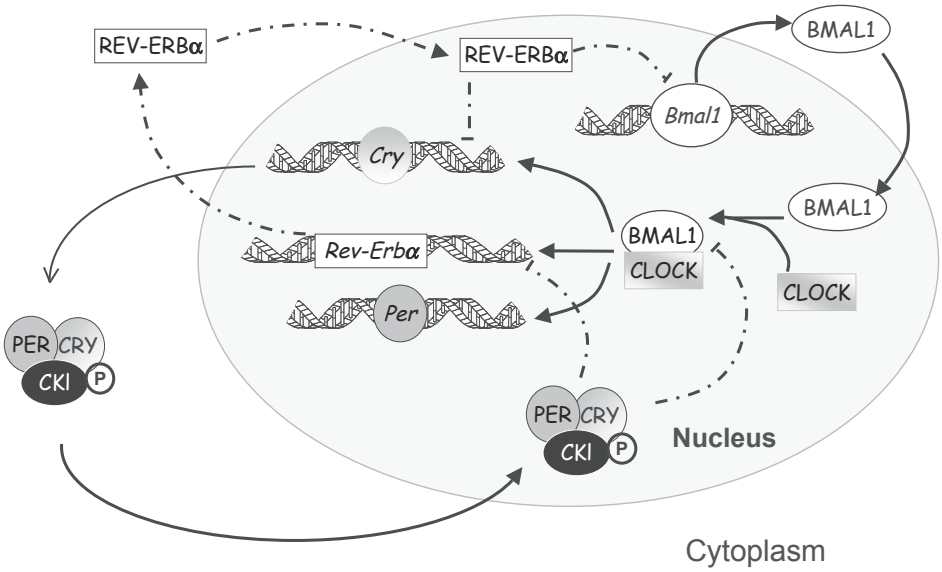

Figure 1 Molecular mechanism of the clockwork. This hypothetical model is based on interacting positive and negative transcriptional/translational feedback loops. The positive feedback loop is mediated by the proteins BMAL1 (expressed in a rhythmic manner with high levels during the dark period) and CLOCK (which exhibits constant levels along the L/D cycle). These two proteins heterodimize and activate the transcription of three *Period* genes (*Per1/2* and *3*), two *Cryptochrome* genes (*Cry1* and *2*) and the *Rev-erb*α gene. PER and CRY proteins then form heterodimers, which are phosphorylated by casein kinase in the cytoplasm, and enter back into the nucleus to repress their own transcription by acting on the BMAL1:CLOCK complex. This step represents the negative feedback loop. The primary loop is modulated by the secondary loop via the REV-Erα, which drives the circadian profile of *Bmal1* gene expression.

2. Organization of the suprachiasmatic nucleus

At present the SCN can be divided into two main parts depending on its projections, input or transmitter content: - The ventrolateral part of the SCN encloses neurons which contain vasoactive intestinal polypeptide (VIP), peptide histidine isoleucine (PHI), and gastrin releasing peptide (GRP). These peptides display a rhythmic mRNA expression driven by the L/D cycle [35]. This part of the SCN receives the terminal arborization of the direct visual afferents from the retinohypothalamic tract (glutamate) and indirect via the intergeniculate leaflet (IGL) of the thalamus, containing neuropeptide Y (NPY) and GABA [8, 36-38]. Moreover, it was recently proposed that also in the blood stream circulating information may come to the SCN via a projection from the arcuate nucleus, which may also contain NPY [39]. - The dorsomedial part of the SCN encloses

neurons containing arginine vasopressin (AVP) and somatostatin (SS) neurons [40]. Not much is known about the input of the dorsal part. The most detailed knowledge is about the input to the ventral part of the SCN [41].

In view of its input, the ventral SCN is proposed to be involved in the processes of light/dark entrainment and to convey this information to the dorsomedial part of the SCN in order to spread the new "adjusted timing signal" [42,43]. This interaction of light-receiving versus not-light-receiving organization of the clock was confirmed by light pulse studies. Light, perceived by the retina, is transmitted to the SCN by a release of glutamate from the retinal terminals [44,45], which in turn increases the neuronal activity of the SCN-VIP cells via NMDA and non-NMDA receptors [46,47]. The fact that light induces Fos expression in the ventral SCN in a different pattern, depending on the circadian time [48-50], indicates that the SCN also gates the light information. This could reflect the mechanisms by which light may induce either phase advance or phase delay in the activity of the SCN. This is supposed to be due to the moment in the clock cycle at which light information affects its movement. In addition, it is also known that light has a different effect on the output of the SCN, depending on the time of the night (or day) it reaches the SCN [51,52]. Whether this takes place via a different phase of clock gene expression in the ventral SCN and/or by the differences in the output of the ventral SCN to the dorsal SCN is not clear at present. At a cellular level, transcription of immediate-early genes (IEG) such as c-fos, egr, jun-b will occur as well as Per1 gene expression [53,54]. Recently, a relationship between Per1 mRNA induction and neuronal spike activity was demonstrated in the VIP neurons of the retino recipient division of the SCN [55].

However, as was to be expected, light exposure does not affect clock-gene expression only in the retino recipient part of the SCN but also in other regions, depending on the time during which the light pulse is applied [56], i.e. a light pulse in the early night triggers an increase of Per1/2 in the ventral part of the SCN and Per2 in the dorsal part, whereas light exposure late at night induces Per1 mRNA in both SCN subdivisions. This regional distribution of light-activated cells indicates that the SCN does not respond in a homogenous manner to light but rather shows a differential cellular activation depending on the time of the light exposure [57]. Considering that many VIP and GRP containing cells are activated and show Fos, and that VIP-containing projections from the SCN reach many brain areas, such as the paraventricular nucleus of the thalamus (PVT), PVN, MPO or DMH [58-61], VIP cells will also convey the light information to areas outside the SCN. The photic input may then influence other brain target areas involved in endocrine/metabolism control via a direct route (VIP efferent) and/or indirect pathways via the VP-containing projections. Recently, studies that focussed on the SCN control of melatonin synthesis also provided information about the organization of the SCN. Indeed, the SCN generates the nocturnal peak of mela-

tonin by using two distinct signals: a rhythmic inhibitory signal driven by GABAergic cells and a continuous stimulatory signal that is probably provided by glutamatergic cells [62, 63]. In addition, the SCN, activated by light, uses (also at night) GABA as an inhibitory transmitter to transmit this light information to the PVN. Both the dorsal and ventral subdivisions of the SCN are proposed to contain neurons responsible for the offset and onset of the melatonin release [63]. Since GABA is present in about 30% of the VP and VIP neurons the light signal to the pre-autonomic sympathetic PVN neurons that inhibit melatonin secretion could be mediated by GABA, co-localized in a small population of VIP neurons.

These studies reveal the SCN as a heterogeneous nucleus in which sets of neurons are differentially activated, depending on the input "stimulus". We think that the SCN forms a unit in which a high density of internal connections takes care of an SCN output that is highly integrated with the light dark cycle and the physiology of the body [39].

3. SCN output and its transmission of light information

The SCN conveys its circadian signal by diverse projections to hypothalamic areas [64, 65]. Three main hypothalamic neuron populations have been identified as the major SCN projection targets:

- **endocrine neurons,** which contain, for instance, gonadotropin-releasing hormone (GnRH), corticotrophin-releasing hormone (CRH) or thyrotropin-releasing hormone (TRH). Through these neuro-endocrine neurons, the SCN controls neuroendocrine functions such as the estrous cycle [66, 67].
- **autonomic neurons**, located in the PVN, connect the hypothalamus with sympathetic and parasympathetic motor nuclei [68-72]. Using this pathway, the SCN drives peripheral tissues and organ functionality, such as the pineal gland for a rhythmic production and release of melatonin [62], or the adrenal, pancreas, liver or fat to control metabolism [51, 73-76].
- **intermediate neurons**, located in MNP, DMH, sPVN, integrate, among other things, the circadian information before it is transmitted to endocrine or autonomic neurons [61, 77, 78].

Light: Short-term effects

As mentioned above, a light pulse not only triggers neuronal firing of SCN cells but also a cellular signalling cascade, resulting in IEG/clock gene transcription. The immediate impact of a light pulse on SCN output, known to affect the autonomic nervous system activity and then the peripheral organs[79, 80], has not been extensively studied, except light-induced inhibition of melatonin synthesis. As mentioned previously, the circadian clock generates synthesis and secretion of melatonin at night. The SCN is

connected to the pineal gland via multisynaptic pathways, i.e. the SCN–PVN–intermediolateral cell column of the spinal cord (IML) - the superior cervical ganglion (SCG) - the pineal gland. In rodents, synthesis and release of melatonin is triggered by the noradrenalin release/sympathetic activation that occur at night. GABA release from SCN terminals during the day silences PVN pre-sympathetic neurons, whereas at night glutamate activates PVN pre-autonomic neurons, resulting in melatonin synthesis. At night, light stimulates SCN neurons, which release GABA in the PVN, resulting in an immediate suppression of melatonin production [62, 81]. In addition to melatonin, light exposure also affects corticosterone secretion during the subjective night period using the multisynaptic pathways; i.e. the SCN is connected to the adrenal cortex by the sympathetic branch of the autonomic nervous system. The light exposure induces an immediate (after 5 min) decrease in corticosterone levels, which, in contrast to melatonin, occurs only when light is applied in the first part of the night [51]. Similarly, it was recently found that longer light exposure results in an increase of corticosterone after 1 hour [82]. In the same way, light at night reduces the heart rate only when a functional SCN is present. Light activates the heart rate in diurnal species such as man, indicating that light, just like melatonin, is used by the SCN to signal the time of the day. This proposal is further strengthened by the observation that animals become immediately (i.e. within 5 minutes) inactive after they have been exposed to light, even if that exposure occurs during their normal activity period [52]. All this is evidence that light induces immediate changes in the output of the SCN, which is transmitted by hormones and the ANS to the body, immediately affecting physiology and behaviour.

Light: Long-term effects

Apart from the fact that light induces an acute increase in SCN electrical neuronal activity and transmitter release from its terminals and IEG and Per expressions in the cell body, light activation can also induce a clock phase-delay or a clock-phase advance when the light pulse is applied early or late at night, respectively [83]. Although there are indications that phase shifts take place immediately [84], their behavioural effects can only be observed after some time, because the light-induced clock resetting will modify the circadian output, leading to a phase-shift in the locomotor activity, for instance [85]. The blockade of light-induced Per1 up-regulation abolishes the phase shift in locomotor activity, suggesting that the Per1 mRNA increase is part of the clock resetting process [86, 87]. Since Per1 mRNA upregulation is followed by an increase in protein levels, the Per1 mRNA increase might create a dysbalance of the clock proteins within the SCN neuron and thereby affect the phase of the clock [25]. If the phase change exceeds the capacity of the immediate phase shift then the adjustment to the new "timing" is not immediate: there is a "transient period" in which behavioural and

physiological rhythms will adapt to the new phase. In case of phase delay, in which the normal cycle is temporarily lengthened, the clock timing can extend in line with its natural/endogenous period (period of about 27 hours) and can thus easily catch up with the new L/D cycle. In addition to the daily synchronization of the clock by light, recently a role of the clockwork in the integration of the photoperiodic changes was proposed for the anticipation of the physiology and metabolism to a new season [88].

As part of the generation of the daily rhythm in behaviour and physiology, the biological clock also orchestrates and synchronizes metabolic processes within the body according to the sleep/wake or rest/activity period, i.e., to promote a more effective metabolism by synchronizing the functional activity of organs to the time of the day.

Since the temporal distribution of food intake is clearly driven by the circadian clock, it has been assumed that the daily rhythms in circulating concentrations of metabolic hormones and substrates (e.g., insulin, glucagon, leptin, glucose and free fatty acids) are induced by the feeding pattern and not via a direct SCN control. However, recently we have provided evidence that the SCN affects hepatic glucose production and glucose uptake by the ANS via the PVN [73, 89]. These and other studies lead us to propose that the SCN also plays a role in anticipating major changes in physiological conditions, i.e. anticipate the glucose demands for the upcoming activity period by means of an increase of corticosterone, a higher glucose tolerance, an increased insulin sensitivity and a higher hepatic glucose production.

Therefore, in the second part of the introduction, we will focus on glucose metabolism and we will give an overview of the mechanisms which may act on glucose metabolism in order to anticipate the glucose demands according to the time of the day.

DAILY RHYTHM IN PLASMA GLUCOSE CONCENTRATIONS

A daily variation of plasma glucose levels has been reported in humans and rodents [5, 90-92]. The 24h-rhythm in plasma glucose level is characterized by a peak which occurs just before the onset of the activity period, i.e. early morning for humans and late day for the rodent. Plasma glucose concentrations are the result of glucose intake (i.e. food absorption from the gut), hepatic glucose production (i.e., gluconeogenesis, glycogenolysis) and glucose tolerance (i.e, glucose uptake by peripheral tissues). In addition, an increase of glucose tolerance is induced by an increase of insulin secretion by the pancreatic β cells (i.e., high insulin response) and/or a higher availability/sensitivity in insulin receptor (i.e, insulin sensitivity) and/or a larger amount of glucose transporters.

1. Glucose rhythm is driven independently of feeding activity

Plasma concentrations in glucose and insulin display a daily rhythm in humans and rodents [5, 93-97]. Since insulin secretion is mainly triggered by an increase of blood glucose due to food absorption, the glucose and insulin rhythms are thought to be indirectly driven by the SCN, via its control over feeding activity. To investigate the direct impact of the SCN on plasma glucose and insulin, fasting and a regular feeding regimen have been used to prevent the influence of feeding behaviour. Under fasting conditions (2-3 days), only plasma glucose levels showed a persistent rhythm [90, 92, 94], suggesting that the rhythmic pattern of plasma glucose is not a consequence of the rhythmic feeding activity. However, starvation as a strategy to remove the rhythmic food signal might not provide the best approach, since obviously the starvation period has to be limited and also because after 2-3 days of starvation the digestive enzyme activity of the small intestine still maintains its rhythm [98, 99]. Therefore, a new strategy in which meals are equally distributed along the L/D cycle (6-meal schedule) was settled in order to disrupt completely the rhythmic feeding signal without affecting the daily locomotor activity. Under this scheduled regimen, plasma glucose levels still display a pronounced 24h-rhythm as found in fasting or *ad libitum* fed animals. Furthermore, SCN-lesions combined with each feeding condition abolished the daily rhythm in plasma glucose concentrations. Together, these studies indicate that SCN controls the plasma glucose levels, independent of the rhythm in food intake [92]. Interestingly, plasma insulin and glucagon concentrations increase equally after every meal in a 6-meal schedule, indicating that pancreatic hormones do not require a rhythm to generate the glucose rhythm [92, 100]. However, the SCN does influence the feeding-induced insulin response, depending on the time of the day [101]. This will be discussed in the next paragraph.

2. Daily rhythms in glucose tolerance and insulin sensitivity

A daily variation in glucose tolerance was demonstrated in humans by administration of an oral or intravenous glucose bolus, i.e., low glucose uptake at the end of the afternoon versus high uptake in the early morning [93, 102-104]. These diurnal changes in glucose uptake were demonstrated also in animal models by using a similar glucose tolerance test [89, 105]. These observations lead to the following question: "How to explain the variations in glucose tolerance?". Theoretically, changes in glucose tolerance could result from a variation in insulin secretion by β-pancreatic cells in response to glucose or by a variation in the sensitivity of the tissue to insulin and/or changes in non-insulin dependent glucose uptake. Human data indicated fluctuations in insulin sensitivity as well as the pancreatic β cells' sensitivity to glucose [93, 103, 104, 106]. However, these data were based on a comparison of morning *versus* evening. Studies performed on a 24h-profile indicated that the diurnal variations in glucose uptake cannot result from a change in insulin response to glucose, but are more likely to be due to a change

in insulin sensitivity [89, 101]. Moreover, SCN-lesion experiments revealed that insulin sensitivity cannot be the only mechanism underlying the diurnal fluctuation in glucose tolerance [89]. Therefore it was proposed that an insulin-independent mechanism of glucose uptake may contribute to the generation of the 24h-rhythm in glucose tolerance. In line with this hypothesis, a recent study performed with cultured primary rat muscle and fat cells showed a circadian rhythm of glucose uptake in muscle and fat by an insulin-independent mechanism [107]. However, no studies have confirmed the rhythmic peripheral glucose uptake *in vivo* yet.

In view of the generation of the daily plasma glucose rhythm, it is clear that the daily rhythm in glucose tolerance is not responsible for the generation of the peak before the onset of the activity period, i.e. the plasma glucose increase occurs at the time of the highest glucose uptake. Therefore the increase of plasma glucose concentration can only be explained by an increase of glucose output (i.e., probably by hepatic glucose production) in order to compensate for the increase in glucose uptake. These observations in rats are in line with the dawn-phenomenon observed in humans, i.e. increased glucose output together with increased glucose utilization [5].

Since endogenous glucose is mainly produced by the liver (i.e., gluconeogenesis and glycogenolysis) [108], we propose that the SCN controls and sends its timing signal to the liver in order to generate the daily 24h rhythm in glucose, i.e., to increase hepatic glucose production at the end of the day, by using hormonal and/or neuronal stimulation.

3. Hormonal *versus* neuronal control of the daily glucose rhythm

Hormonal stimulation of hepatic glucose production

Glucagon is a hormone secreted by the α–pancreatic cells in response to low glucose concentrations in the blood or to hypoglycaemia [109, 110]. It activates hepatic gluconeogenesis by increasing the gene transcription of the phosphoenolpyruvate carboxykinase (Pepck) [111] and stimulates the breakdown of glycogen stored in the liver by activating the enzymes that depolymerize glycogen and release glucose [112].

The connection between the SCN and the pancreas via the ANS [65] as well as a daily variation in plasma glucagon levels in *ad libitum* fed animals [100, 113] indicate glucagon as a potential circadian signal for driving the 24h-rhythm in glucose. However, there are two main reasons why it is unlikely that the daily pattern of plasma glucagon concentrations is responsible for the daily glucose rhythm. Firstly, the peak in plasma glucagon level does not coincide with the peak in plasma glucose level, i.e. the rise in glucose occurs three hours earlier in *ad libitum* fed animals. Secondly, a scheduled feeding regimen abolishes the glucagon rhythm without affecting the daily variation in plasma glucose levels [100].

Insulin, which has an effect that is the opposite of that glucagon on the liver, also cannot explain the daily variation in plasma glucose (see the previous paragraph).

EPINEPHRINE/GROWTH HORMONE/CORTICOSTERONE

Hepatic glucose production can be stimulated by catecholamines [114, 115], growth hormone [116, 117] and corticosterone [118, 119]. However, the ultradian rhythm in growth hormone [120, 121] and the temporal fluctuations of noradrenaline/adrenalin [122] cannot explain the generation of the 24-h glucose rhythm. Corticosterone is still seen as a candidate for driving the daily changes in plasma glucose, probably because the corticosterone peak levels in the blood coincide so well with the peak levels in glucose, even though blocking the corticosterone synthesis did not affect the morning rise in plasma glucose in human [123, 124]. Furthermore, in rats, despite large increases in plasma corticosterone levels, no increase in plasma glucose concentration levels was observed [73].

FAT-DERIVED HORMONES/SUBSTANCES

Besides glucose, free fatty acids (FFA) are also an important fuel used by many tissues. FFA can be produced by adipose tissue (i.e., lipolysis which breaks down triglycerides (TG) in glycerol and FFA) and by the liver. Following food absorption, the increase of plasma glucose levels triggers insulin secretion, which in turn inhibits the production by the liver of FFA and hepatic gluconeogenesis, and increases glucose uptake [125]. With high levels of FFA (due to fasting or physical exercise), fatty acids inhibit the effect of insulin, causing an increase of HGP [126]. The tight interaction between FFA and glucose (the glucose fatty acid cycle) is based on competition [127]: fatty acids inhibit glucose oxidation and cause a switch to gluconeogenesis and glycogenolysis in hepatocyte [126, 128, 129]. Therefore, it makes sense to investigate a possible role of lipid metabolites/hormones in the generation of the 24h-rhythm in plasma glucose levels.

Interestingly, rhythms have been reported in plasma FFA concentrations [113, 130], in fatty acid synthesis in fat and liver [131, 132] and in enzyme activities involved in lipid metabolism [133-137], suggesting a role of lipid metabolism in the generation of the 24h-rhythm in glucose.

White adipose tissue is no longer considered as just a passive organ (i.e., storage of fat) but more and more as an important endocrine organ that participates in body homeostasis by secreting "adipokines" or fat-derived hormones [138].

Leptin is expressed mainly in adipocytes and secreted in the blood under the control of the SCN. We recently demonstrated a rhythm in leptin secretion, which is dependent on the SCN and which is affected by the autonomic output of the hypothalamus [139]. Leptin effects on body weight regulation and metabolism are mediated through hypothalamic centers, which control feeding activity, body temperature and energy expenditure [140]. Leptin may also play an important role in the modulation of metabolic flux and insulin action [141, 142]. Among other things, the central action of leptin can promote hepatic glucose production via gluconeogenesis and glycogenolysis [143]. However, we have to keep in mind that in these studies mostly leptin is used, in pharmacological doses. In addition, initially it was proposed that leptin would be responsible for the

inhibition of food intake, since it is secreted into the blood at levels proportional to the amount of fat. Moreover, the injection of leptin into obese animals that are unable to produce leptin results in an inhibition of food intake, and hence a reduction in weight. Two important but often neglected arguments argue against this: the normal 24 hour peak in leptin occurs at beginning of the dark phase in animals as well as humans and in rat this coincides with the onset of food intake. In addition, in normal overfed obese animals and people high leptin levels can be found without any inhibitory effects on food intake. To "explain" this phenomenon the term "leptin insensitivity" has been introduced. Currently it is assumed that low levels of leptin have a more important signalling function than high levels, e.g. its function is more associated with starvation. This in fact has lead to the proposition that leptin is a signalling molecule of fasting conditions rather than the molecule of surplus.

Adiponectin, like leptin, is secreted by adipose tissue and exerts some of its weight reduction effects via the brain. However, in contrast to leptin, adiponectin inhibits endogenous glucose production [144]. Both leptin and adiponectin display a day/night rhythm in humans and rodents in opposite phases, i.e, decline of plasma adiponectin during the day reaching a nadir in early morning and a rise in leptin levels before awakening [139, 145-148]. Despite a quite similar daily rhythm between adiponectin and glucose no clear evidence indicates a role of this fat-derived hormone in the generation of the glucose rhythm.

Neuronal stimulation of hepatic glucose production

The liver is highly innervated by both branches of the autonomic nervous system, i.e., parasympathetic and sympathetic. The hypothalamus can be seen as the 'head' ganglion of the autonomic nervous system and the role of the hypothalamus in glucose metabolism was discovered by electrical or chemical stimulation in different hypothalamic areas. Activation of the ventromedial hypothalamus produces hyperglycemia by increasing glycogenolysis and gluconeogenesis by the sympathetic efferents directly to the liver and/or via a release of stimulatory hormones such as glucagon [149-151]. Contrary to this, stimulation of the lateral hypothalamus produces hypoglycemia by activation of glycogen synthesis and inhibition of gluconeogenesis due to parasympathetic activation of the liver [152, 153]. Furthermore, using intracranial injection of 2-deoxy-D-glucose (2DG), an essential role of the SCN in glucose metabolism was demonstrated: the hyperglycemic response triggered by 2DG showed a time dependency and in SCN-lesioned animals the increase in plasma glucose levels was completely abolished [79, 80]. Later, retrograde tracing studies using a pseudorabies virus (PRV) as a transsypnatic tracer demonstrated connections between the circadian clock and the liver via the autonomic nervous system [154], suggesting that the SCN can control glucose metabolism via the liver in a circadian manner in order to generate the 24h rhythm

in plasma glucose levels. Therefore we proposed that the SCN sends its timing signal to the pre-autonomic neuron, localized in the PVN, to activate in a differential manner the two autonomic routes and thereby control the hepatic glucose metabolism [155]. In line with this hypothesis, the blockade of the GABA inhibitory SCN input to the PVN by infusion of a GABA-antagonist during the day results in an increase of plasma glucose levels. Interestingly, this hyperglycemia is completely abolished after a sympathetic denervation of the liver, suggesting that the SCN can elicit an increase of plasma glucose levels (i.e., probably by an increase of hepatic glucose production) by activation of the sympathetic innervation of the liver [73].

Therefore we propose that the SCN generates the daily rhythm in plasma glucose by controlling, in a rhythmic manner, the liver enzymes involved in glucose metabolism.

4. Peripheral clock genes: a tool to affect glucose metabolism?

Clock genes, the essential components of the neuronal SCN clockwork, have been reported to be present in peripheral tissues from isolated cells (e.g. epithelials cells, osteoblast, blood mononuclear cells) to organ levels such as the pineal gland, adrenal, thymus, heart, lung, liver, pancreas, skeletal muscle, kidney, fat tissues and spleen [156-165]. The existence of peripheral oscillators has lead to a reconsideration of the organization of the circadian timing system: the SCN master clock orchestrates the circadian oscillations within the organs to drive tissue-specific outputs synchronized with the L/D cycle [65, 166, 167].

Synchronization of the liver oscillator

Feeding activity is proposed to represent the dominant Zeitgeber for synchronizing rhythmic clock gene expression in peripheral tissues such as liver [168]. In fact, restricted feeding can restore and synchronize rhythmicity of peripheral organs and even a behavioural rhythm in SCN-lesioned animals [169-173]. Furthermore, uncoupling the L/D signal and food availability leads to a separation of the phase of the central clock and the peripheral oscillator, reinforcing the idea that feeding activity is a more powerful Zeitgeber than the circadian clock [168]. Thus, when light information and food availability are decoupled, the peripheral oscillators are more flexible than the SCN and are reset by a complex of signalling cues related to pre- and postprandial mechanisms [174-177]. Since the liver is the main organ involved in glucose metabolism (release or storage of glucose), it is not surprising that all nutrients/substances or humoral signals that can be linked to pre- and postprandial processes (such as metabolized nutrients, hormonal release) are able to reset the liver oscillators. However, some evidence indicates that also the central clock can reset the liver oscillator, by controlling humoral signals [178]. Among them, corticosterone represents a potential resetting-signal for the liver clock,

since its 24h rhythm is driven by the SCN [179], whereas also the sympathetic branch of the autonomic nervous system provides a direct pathway to transmit SCN-timing signal [180]. Despite the important role of the "metabolic signal" provided by food intake, the light/dark signal conveyed by the circadian clock still remains an important signal for the synchronization to the liver [181]. For that reason, we propose that under "normal feeding conditions" (i.e., ad libitum), the central clock entrains peripheral oscillators to generate circadian tissue-specific outputs in order to drive, in a precise manner, the daily physiology according to the sleep/wake cycle [65, 82, 182-184].

Circadian gene regulation of enzymes involved in glucose metabolism

The liver plays an important role in glucose metabolism through a delicate balance between glucose uptake (i.e., storage by glycogenesis) and glucose release (i.e., glycogenolysis and gluconeogenesis). Hepatic glucose release/uptake is controlled by Glucose-6-phosphatase (G6Pase) and glucokinase, respectively [185]. Hypoglycemic signals trigger an up regulation of phosphoenolpyruvate carboxykinase (Pepck) and glycogen phosphorylase to increase glucose output via gluconeogenesis and glycogenolysis, respectively. At the same time, inhibition of pyruvate kinase and glycogen synthase will occur to decrease glycolysis and glycogen synthesis, indicating a tight link in the regulation of mechanisms involved in glucose storage/use and hepatic glucose production [108] (Figure 2). Interestingly, it is indicated that hepatic glucose metabolism displays a daily fluctuation by means of a rhythmic gene expression in glucokinase, glycogen synthase and Pepck [186-189]. Furthermore, recent studies demonstrated a direct control of the liver clock gene on glucose-metabolizing genes, reinforcing the idea that the liver oscillator might play a role in liver metabolism [188-191]. The present thesis therefore investigates whether the liver oscillator drives the metabolizing-glucose enzyme in a circadian manner, rendering a more effective glucose metabolism by synchronizing the functional activity of organs with the time of day, both through neuroendocrine mechanisms and the autonomic nervous system (ANS).

SCOPE OF THE THESIS

The control of glucose homeostasis is based on a multifaceted mechanism, i.e. all tissues, pancreatic hormones, fat-derived hormones, the autonomic nervous system, and the liver, play a role in glucose uptake and production. In the present thesis we focus on the role of the autonomic nervous system (ANS) and the liver in glucose homeostasis. One of the strategies to maintain glucose homeostasis is the anticipation of foreseen changes in the environment. One of the clearest examples is the anticipation to seasonal changes. Here, the SCN-pineal axis plays an essential part in preparing

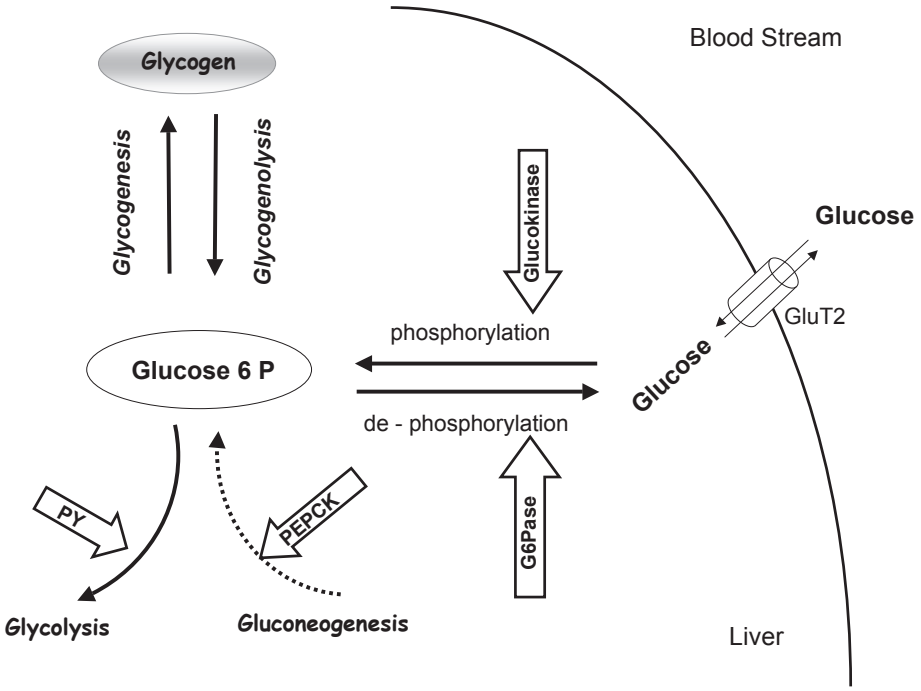

Figure 2 Key enzymes involved in hepatic glucose metabolism.

the animal for the upcoming changes in food supply, resulting in profound changes in reproduction and metabolism. A number of species are less subject to seasonal changes but in all mammals the SCN has a special role in the anticipation to daily changes such as the approach of the active period of the organism, human or animal. This anticipatory role of the SCN is characteristic for its involvement in the organization of the physiology of the animal and its influence on metabolism, by means of a rise in glucose, corticosterone and body temperature just before awakening. Since the appropriate organization of metabolism (in particular the regulation of glucose homeostasis) is vital importance for an organism, we examined in the present thesis the role of the SCN and its control in the ANS input to the liver for the rhythm in plasma glucose levels. The thesis can be divided in two parts:

First part

Recently it was shown that the activation of the sympathetic input to the liver can trigger an increase in plasma glucose concentrations, suggesting a role of this autonomic route in the daily peak in plasma glucose levels. Therefore, in the first part of the thesis, we investigated whether the sympathetic innervation of the liver is involved in the genesis of the 24h-rhythm in plasma glucose levels. In addition, in view of the

importance of clock genes for the rhythmic electrical activity in the SCN, we investigated whether clock genes and the glucose-metabolizing enzyme, which are both rhythmically expressed in the liver, are responsible for the genesis of the 24h-rhythm in plasma glucose concentrations (Chapter 2/3). Having observed the lack of contribution of the clock genes to the rhythm of liver enzymes (i.e. sympathetic denervation did affect the rhythmicity of liver enzymes but not that of the clock gene), we decided to examine the contribution of the ANS and food intake to the rhythmicity of liver enzymes and blood glucose levels (Chapter 3).

Second part

We aimed to investigate how the SCN controls autonomic inputs to the liver in order to reset the liver clock gene and thereby modifies or adjusts the glucose metabolism according to the time of the day. Recently, we have shown that the inhibitory transmitter GABA mediates the light-induced inhibition of the rise in plasma glucose levels via the sympathetic preautonomic neurons in the PVN. This observation led us to identify whether the activation of the sympathetic input affects the clock gene expression in the liver and the enzymes involved in glucose metabolism. Since GABA-antagonist infusion in the PVN results in activation of all preautonomic neurons, i.e. increases in glucagon and corticosterone, we were able to identify a selective effect of the sympathetic input on the liver clock-gene and enzyme transcripts (Chapter 4). Therefore we aimed at using a more specific approach to affect the autonomic output of the PVN. Since light immediately affects the output of the SCN, and in addition resets the time of the SCN, both processes may in turn modify the physiology and metabolism according to the new time of the day. We therefore decided to investigate the role of the ANS in the transmission of the light signal to the liver. By using complete autonomic denervation of the liver we could dissect autonomic from hormonal input and examined how the autonomic input influences the liver clock genes and glucose-metabolizing enzymes in order to immediately respond to an environmental change (Chapter 5).

Chapter 2

The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved?

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Abstract

In order to drive tissue-specific rhythmic outputs, the master clock, located in the suprachiasmatic nucleus (SCN), is thought to reset peripheral oscillators via either chemical/hormonal cues or neural connections. Recently, the daily rhythm of plasma glucose (characterized by a peak before the onset of the activity period) has been shown to be directly driven by the SCN, independently of the SCN control of rhythmic feeding behavior. Indeed, the daily variation of glucose was not impaired unless the scheduled feeding regimen (6-meal schedule) was associated with an SCN lesion. Here we show that the rhythmicity of both clock gene mRNA expression in the liver and plasma glucose is not abolished under such a regular feeding schedule. Since onset of the activity period and hyperglycemia are correlated to an increased sympathetic tonus, we investigated whether this autonomic branch is involved in the SCN control of plasma glucose rhythm and liver rhythmicity. Interestingly, hepatic sympathectomy combined with a 6-meal feeding schedule results in a disruption of the plasma glucose rhythmicity without affecting the daily variation of clock gene mRNA expression in the liver. Taking all these data together, we concluded that i) the SCN needs the sympathetic pathway to the liver to generate the 24h-rhythm in plasma glucose concentrations, ii) rhythmic clock gene expression in the liver is not dependent on the sympathetic liver innervation and iii) clock gene rhythmicity in liver cells is not sufficient for sustaining a circadian rhythm in plasma glucose concentrations.

Introduction

In mammals, the suprachiasmatic nucleus (SCN) synchronizes behavioral and physiological rhythms to the day/night cycle via its efferent projections [71, 192, 193]. SCN neurons exhibit a circadian oscillation of electrical activity driven by a molecular mechanism based on interacting positive and negative transcriptional-/translational-feedback loops. At least seven clock genes play a crucial role in the clockwork: *Bmal1*, *Clock*, part of the positive limb, and *Cry1/2*, *Per1/2/3*, part of the negative limb [25, 194, 195].

Clock genes have been demonstrated throughout the entire body [161, 196]. The discovery of these peripheral oscillators has led to a reconsideration of the organization of the circadian system. Many studies (e.g. SCN lesions, culture explants) have provided evidence that the peripheral oscillators need the master clock to maintain their rhythmicity [197, 198]. However, other reports showed that peripheral organs possess a self-sustained oscillator [199, 200], suggesting that the SCN controls the tissue-specific outputs by synchronizing the peripheral oscillators [199]. Nevertheless, it remains unclear how the SCN synchronizes peripheral oscillators. Moreover, the question whether the peripheral circadian clockwork drives rhythmic output of gene expression within tissues, resulting in organ specific function, has not yet been answered.

It is known that the SCN conveys its "timing" signal to the periphery via different routes: neural connections (autonomic branches), hormonal cues (e.g. corticosterone, melatonin, growth hormone) and rhythmic behaviour [65, 178]. For the liver, studies revealed that chemical cues (e.g. glucocorticoids, adrenaline) or sympathetic nerve stimulation can either elicit a phase shift or trigger rhythmic gene expression [179, 180]. Also, restricted feeding appears to have a powerful effect on resetting clock gene expression in the liver [168, 173]. Interestingly, glucose metabolism, too, appears to be affected by hormonal cues, autonomic activity and feeding behaviour [201].

In search of a functional role for clock gene expression in the liver, this evidence triggered our interest in the regulation of liver clock genes in relation to glucose metabolism. Starting from the hypothesis that hepatic glucose production and the liver clockwork are associated, we explored how the SCN synchronizes liver metabolism and rhythmic clock gene expression. Since food intake affects clock gene expression [173] and plasma glucose rhythm is not disturbed by scheduled feeding [92] we first monitored the daily expression of liver clock genes under this scheduled feeding regimen. Subsequently we investigated the role of the sympathetic liver innervation in the control of the plasma glucose rhythm and clock gene rhythms, since this autonomic branch is known to increase plasma glucose concentration (most likely via an increase of hepatic glucose production) and clock gene expression in the liver [73, 180, 202].

Materials & Methods

Animals

Adult male Wistar rats (Wu, Harlan, Zeist, The Netherlands) were kept in a 12 h light/12 h dark cycle (L/D; lights on at 7:00, considered as Zeitgeber time (ZT) 0) under constant conditions of temperature (20 ± 2°C) and humidity (60 ± 2%) with water *ad libitum*. All experiments were conducted with the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Six- meal regimen

Rats were entrained to a regular feeding schedule consisting of six meals spread equally over the L/D cycle. Food pellets (Global Rodent Breeding, purified pellet, Harlan Teklad) were available every 4 hours (ZT2, 6, 10, 14, 18 and 22). The access to food was 11 and 9 minutes for the day-time and night-time meals, respectively. The rats were given three weeks to adapt to the feeding schedule. The amount of food consumed during the day- and night-time meal was measured regularly. During the third week of adaptation, the mean size of a day-meal was 3.44 ± 0.05g and for a night-meal 3.27± 0.16g (n = 32 animals). For more details on the six-meal schedule, see [101, 139].

Surgical procedure

GENERAL PROCEDURE

Surgery was performed in rats of 300-350g. Rats were anesthetized using a mixture of Hypnorm (Janssen, 0.05ml/100g BW, i.m.) and Dormicum (Roche Nederland, 0.04ml/100g BW, s.c.). During surgery, the abdominal cavity was bathed regularly with saline to prevent drying of the viscera. The wound was closed with atraumic sutures (5-0 Perma-Hand Said Ethicon). After the surgery, the animals were placed in an incubator until awakening (temperature 30°C); saline was injected subcutaneously to prevent dehydration. Post-operative care was provided by subcutaneous injection of the painkiller Temgesic (Schering-Plough (Utrecht importer), 0.01ml/100g BW), after the operation and on the following day. Animals were allowed to recover for two weeks and subjected to either a six-meal schedule or allowed *ad libitum* food access, according to their experimental group.

HEPATIC SYMPATHECTOMY

A laparotomy was performed in the midline. The liver lobes were gently pushed up and the ligaments around the liver lobes severed to free the bile duct and the portal vein complex. The bile duct was isolated from the portal vein complex. At the level of the hepatic portal vein, the hepatic artery, a branch of the celiac artery, divides into the hepatic artery proper and the gastro-duodenal artery. This cleavage occurs on the ventral surface of the portal vein. At this point, the arteries were separated via blunt dissection from the portal vein. The nerve bundles running along the hepatic artery proper were visualized using a myelin-specific dye (Toluidin Blue) and were removed using micro-surgical instruments under an operating microscope (25x magnification). Any connective tissue attachments between the hepatic artery and the portal vein were also broken, eliminating any possible nerve crossings. The sympathetic denervation involves an impairment of both efferent and afferent nerves. However, this procedure does not impair the vagal input to the liver, as shown previously [73].

JUGULAR VEIN CATHETER

During the same surgical procedure (see above), an intra-atrial silicone cannula was

implanted through the jugular vein according to the method of Steffens [203] for all the groups whose glucose, insulin and corticosterone were measured.

Experimental Set-up

EFFECT OF SIX-MEAL SCHEDULE ON LIVER CLOCK-GENE EXPRESSION

It has been shown previously that our scheduled feeding does not impair the daily rhythm of plasma glucose concentrations [92]. In the first part of our study we checked whether, under the same feeding conditions, the clock gene expression profile in the liver was affected or not. Therefore, groups of rats either had *ad libitum* access to food or were entrained to the six-meal regimen. After the required period of synchronization, the animals were sacrificed at ZT 0.5, 6.5, 12.5, 18.5 (n = 5 per point).

EFFECT OF HEPATIC SYMPATHECTOMY (HSX) ON PLASMA GLUCOSE RHYTHM UNDER SCHEDULED FEEDING CONDITIONS

After three weeks of entrainment to the scheduled feeding regimen, the animals were divided into two groups: sham-operated (n = 8) or HSx (n = 17). All animals were fitted with a jugular vein catheter in order to be able to follow plasma profiles of glucose, insulin and corticosterone. One week after surgery, animals of both groups consumed the same amount of food as prior to the surgery (± 3g per meal). After two weeks of recovery, 0.2ml of blood was collected once every hour during 12 consecutive hours, on two different occasions within a period of two weeks. The two runs started at ZT6.5 and ZT18.5. After blood sampling the completeness of sympathetic liver denervation was checked using transneuronal virus tracing (the Bartha strain of the Pseudorabies virus). Pseudorabies virus was injected into the left lobe of the liver under anesthesia (described in the general procedure of the surgery), containing 5 x 10^6 plaque-forming units. The rats were allowed to survive 5 days, to give the virus maximal time for infection. In sham-operated rats the first neurons become visible in the CNS after 2 days of survival time in the sympathetic preganglionic neurons, located in the intermediolateral column (IML) of the spinal cord. No infection after 5 days survival time should then confirm the quality of the sympathetic denervation. The virus was visualized by means of immunocytochemistry, as previously described [154].

EFFECT OF HEPATIC SYMPATHETIC DENERVATION ON LIVER CLOCK GENE EXPRESSION UNDER SCHEDULED FEEDING CONDITIONS

Rats were divided into three groups: two groups, one non-operated (n = 6 per time point) and one sham-operated (n = 4 per time point), had free access to food; the third group consisted of hepatic sympathectomized rats subjected to the six-meal regimen (n = 5 per time point). One week after surgery, animals subjected to the six-meal feeding schedule consumed the same amount of food at each meal as before the surgery (± 3g per meal). After two post-operative weeks, groups of animals were sacrificed every four hours along the L/D-cycle (ZT2, 6, 10, 14, 18 and ZT22). The completeness

of the hepatic sympathectomy was verified by measuring the noradrenaline content of the liver by HPLC analysis, as the virus treatment is not compatible with RNA extraction.

High Performance Liquid Chromatography-Electrochemical Measurements

For verification of the completeness of sympathectomy, sections of liver were taken after experimental procedures and processed using High Performance Liquid Chromatography (HPLC) with electrochemical detection for noradrenaline (NA) content.

Tissue samples were weighed and homogenized in ice-cold 0.1M perchloric acid (2ml/100mg) and centrifuged (14000 rpm) for 15 minutes at 4°C. The aliquots were then transferred to clean Eppendorf ™ tubes and centrifuged again. Samples of 20µl supernatant were subsequently transferred to the HPLC for analysis (Waters 600E pump and Waters 717plus autosampler, Waters Chromatography b.v., Netherlands; Decade VT-03 electrochemical detector, Antec Leiden, Netherlands and Shimadzu Class-vp ™ software v.5.03 Shimadzu Duisburg, Germany).

The mobile phase consisted of 0.06mol/l Sodium Acetate, 9mmol/l Citric acid, 0.37mmol/l Heptasulfonic acid and 12.5% methanol. The flow rate was kept constant at 0.3ml/min. Separation of NA from other endogenous compounds was achieved with a Supelcosil column (LC-18-DB 25cm x 4.6mm x 5 µm), with a 2 cm guard-column of the same material (Supelco Superguard ™, Supelco USA), column temperature was kept constant at 28°C. Quantification was achieved by means of electrochemical detection (+0.65V) and NA content was measured against a calibration curve of an external NA standard. The detection limit (i.e. signal to noise ratio = 2) was 1 pg of NA injected on the column.

Activity measurements

Locomotor activity recordings were carried out using an analog Piezo-electric stabilimeter, the signals of which were transmitted to a PC-based analog computer interface (CED 1404 Cambridge Electronic Design LTD). The rat cages were placed on a baseplate which in turn was placed on 4 parallel-connected Piero-electronic sensors (Murata, 27mm round disks). The voltage output of the sensors is proportional to relative changes in pressure, i.e. activity of the rat. The activity signals were transmitted directly to the analog inputs of the interface and, with the help of relevant software, transformed into absolute values, i.e. activity bouts. They were added up over a period of 5 minutes and stored into text files for later analysis.

Plasma measurements

Plasma glucose concentrations were determined in triplicate using a Glucose/GOD-Perid method (Boehringer Mannheim, GmGH. Germany). Plasma immunoreactive

insulin concentrations were determined in duplicate with a radio immuno-assay kit (Linco Research, St. Charles, MO). The lower limit of the assay was 0.2ng/ml and the coefficient of variation of the immunoassay was less than 8 %.

Plasma corticosterone concentrations were determined in duplicate with a radio-immuno-assay kit (ICN Biomedicals, Costa Mesa, CA). From the samples, 10µl was taken and diluted in 4 ml of assay buffer. The lower limit of the assay was 1ng/ml and the coefficient of variation of the immunoassay was less than 4 %.

Rat liver RNA extraction and cDNA synthesis

Total RNA was extracted from each liver collected at the different ZT time points by RNeasy mini Kit (Qiagen, Courtaboeuf, France) and included DNase step, according to the manufacturer's instructions.

CDNA SYNTHESIS

2µg of each total RNA was reverse-transcribed in a final reaction volume of 20µl. First, total RNA and a random hexamer (125ng/µg of RNA, Hexanucleotide Mix, Roche, Life Technology Nederland) were incubated for 10 min at 70°C. Then, 5X first strand buffer, 0.1M DTT and dNTPs (10mM) were added and the samples were incubated for 2 min at 42°C. Finally, 1µl of Superscript II RNase H^- Reverse transcriptase (SSIIRT, In Vitrogen, Life Technology Nederland) was added and the reaction was incubated for one hour at 42°C. In parallel, as a control (to detect any unspecific amplification for each sample), 2 µg total RNA underwent the same procedure, except that 1µl of SSIIRT was replaced by 1µl of water (No Reverse-Transcriptase or No RT). Each single-stranded cDNA and its respective No RT was then used as a template for the Real-Time PCR.

Real-Time PCR (RT-PCR)

Five clock genes [Period (*Per*) 1/2/3, Cryptochrome (*Cry*) 1 and D site of albumin promoter-binding (*Dbp*, a clock-controlled gene)] and two reference genes [(ubiquitin conjugate enzyme (*Ubc2e*) and TATA box binding protein (*Tbp*)] mRNA levels were analyzed by RT-PCR.

SET-UP OF THE TECHNIQUE

Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primer data are given in Table 1.

One µl of each cDNA was incubated in a final volume of 20µl RT-PCR reaction containing 1X SYBR-green master mix (Applied Biosystems) and 6 pmol (reverse + forward) of each primer. Quantitative PCR was performed in an Applied Biosystems (Model ABI5700 Prism Sequence Detection System). Cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C; 1 min at 60°C. The data were acquired and processed automatically by Sequence Detection Software

Table 1 Gene nomenclature, GenBank accession code, primer sequences and primer efficiency

Gene	GenBank	Forward Primer	Reverse Primer	Efficiency
Cry1	NM 198750	AAGTCATCGTGCGCATTTCA	TCATCATGGTCGTCGGACAGA	1.98
Per1	XM 340822	TCTGGTTCGGGATCCACGAA	GAAGAGTCGATGCTGCCAAAG	1.84
Per2	NM 031678	CACCCTGAAAAGAAAGTGCGA	CAACGCCAAGGAGCTCAAGT	2.2
Per3	NM 023978	ATAGAACGGACGCCAGAGTGT	CGCTCCATGCTGTGAAGTTT	2.1
Dbp	NM 012543	CCTTTGAACCTGATCCGGCT	TGCCTTCATGATTGGCTG	1.96
Tbp	XM 217785	TTCGTGCCAGAAATGCTGAA	TGCACACCATTTTCCCAGAAC	1.89
Ubi	U 13176	CTCCAACAGGACCTGCTGAAC	CTGAAGAGAATCCACAAGGAATTGA	2.04

(Applied Biosystems Inc). Preliminary experiments were performed to establish the amplification efficiency (E) for each of the primer pairs (to allow direct comparison of the expression levels of the different clock genes throughout the L/D cycle) and the optimal dilution of the cDNA to obtain a cycle threshold (Ct) between 15 and 30 cycles, as recommended by the manufacturer. For all primer pairs the amplification efficiency (E) was determined on the basis of a cDNA dilution series. The Ct values obtained from the dilutions are expressed as the logarithm of the dilution factor: Ct = log(dilution factor). The slope of the best-fit line allowed us to determine the efficiency E according to the following equation: $E = 10^{-(1/\text{slope})}$ (Table 1). Primer specificity was confirmed by dissociation (melting) curve analysis and agarose gel electrophoresis. To correct the cDNA load for the different samples, the target gene had to be normalized by a reference gene. Reference genes are selected by their constant expression throughout the L/D-cycle. The selection of the two reference genes used in our studies, Ubc2e ubiquitin conjugate enzyme and TATA box binding protein, was confirmed by the GeNorm v3.3 program (http://allserv.ugent.be/~jvdesomp/genorm/).

DETECTION OF CLOCK GENE EXPRESSIONS BY THE RT-PCR

The levels of mRNA of five clock genes (Per1/2/3, Cry1 and Dbp) in each sample collected throughout the L/D-cycle were quantified. The amount of each transcript was first normalized independently, with the average calculated from the transcript levels along the L/D. The value obtained was then divided by the average of the normalized values of the two reference genes. Control PCRs were performed using PCR reaction mix, including primers without cDNA (non-template control) or with 1µl of the No RT reaction in the reaction mixture.

Euthanasia

For the clock gene expression measurements, animals were sacrificed by decapitation. To minimize RNase activity within the tissue, pieces were collected and directly frozen into nitrogen and kept at –80°C until analysis. In these animals, the complete-

ness of the denervation was determined by analyzing the noradrenaline (NA) content in the liver by HPLC. PRV-injected rats were sacrificed 5 days after injection. They were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (Nembutal®, Ceva Sante Animal B.V, Maassluis, Nederland) and perfused through the left ventricle of the heart with a saline solution followed by a solution of 4% paraformaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline (0.1M Phosphate-buffered saline, pH 7.2).

Statistical analysis

GLUCOSE, CORTICOSTERONE AND INSULIN PROFILES

The plasma concentrations of glucose, corticosterone and insulin are expressed as mean ± SEM. Statistical analysis was conducted using a repeated-measures analysis of variance (ANOVA) to test for an effect of time. If ANOVA detected a significant effect of time, a cosinor analysis was performed, using the fundamental period (24h) for the individual sets of data points. Curve fitting was performed using constrained nonlinear regression analysis (with the SPSS Advanced statistic 11.0). Parameters for which the significant level of the fitted curve was less than 0.05 were used for further calculation of parameter means and examination of modulation of the rhythm throughout the L/D cycle [204]. The fitted function is defined by its mesor (rhythm-adjusted mean), amplitude (50% of the difference between the maximum and the minimum), and acrophase (time of the maximum). ANOVA and the Student's t-test were used to detect significant differences between sham-operated and HSx rats. For the ANOVA, (paired) t-tests and the cosinor analysis, $p<0.05$ was considered to be a significant difference. In all cases, statistics and cosine analysis were done on absolute values.

RT-PCR DATA ANALYSIS

Data from all experiments are presented as mean ± S.E.M. To demonstrate a statistically significant difference among the different ZT time-points, the P-value of One-way ANOVA was calculated on the basis of the normalized data (SPSS package V12.0). When the ANOVA analysis indicated such a difference, post hoc t-tests (paired) were performed with a significance set at 0.05. To evaluate an effect of groups (control / experimental groups), MANOVA was performed with a significance set at 0.05. The student t-test (un-paired) was applied to estimate a significant difference between two groups at specific ZT points.

Results

Effect of six-meal schedule on clock-gene expression in the liver

Under *ad libitum* conditions, the clock gene mRNA expression showed a rhythmic pattern ($p\leq 0.001$) in accordance with data previously described in mouse and rat [168, 170, 205-207]. In the six-meal schedule too, all clock genes showed a clear daily expression

rhythm ($p \leq 0.001$). The transcript levels of the five clock genes in both groups showed a variation along the L/D cycle with higher nocturnal values: a peak at ZT12.5 for Per1/3 and Dbp mRNA and a peak at ZT18.5 for Cry1/Per2 mRNA (Figure1, Table 2).

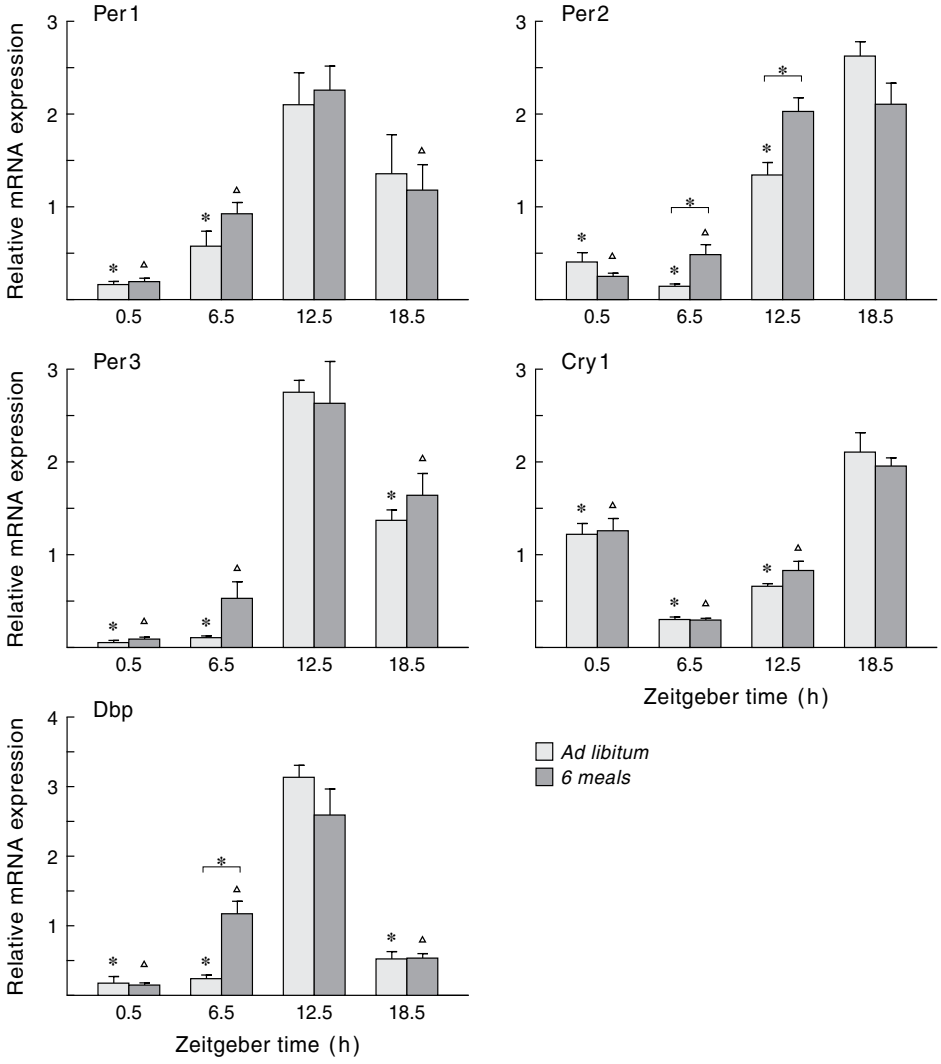

Figure 1 Circadian variation in Per1, Per2, Per3, Cry1 and Dbp mRNA levels in the liver. Animals were housed in 12h light/12h dark cycle with lights on at 07:00 under *ad libitum* condition or under 6-meal schedule, and were sacrificed (n = 5) at the indicated times of the daily cycle. The peak of mRNA expression for each clock-gene was compared with that of other time points; $^{*}P > 0.05$ for *ad libitum* group, $^{\Delta}P < 0.05$ for 6-meal schedule group. A bracket with asterisks indicates a time point in which the two experimental groups (*ad libitum* versus 6-meal schedule) differ significantly from each other.($^{*}P < 0.05$).

Table 2 Statistical analysis of the diurnal variations of clock gene mRNA expression in intact rats under *ad libitum* and scheduled feeding regimen.

			Two-way ANOVA		
		Single	**Time (T)**	**Group (G)**	**Interaction (G x T)**
Per1	Ad libitum	≤ 0.001	≤ 0.001	0.606	0.752
	6 meals schedule	≤ 0.001			
Per2	Ad libitum	≤ 0.001	≤ 0.001	0.353	≤ 0.001
	6 meals schedule	≤ 0.001			
Per3	Ad libitum	≤ 0.001	≤ 0.001	0.288	0.544
	6 meals schedule	≤ 0.001			
Cry1	Ad libitum	≤ 0.001	≤ 0.001	0.866	0.534
	6 meals schedule	≤ 0.001			
Dbp	Ad libitum	≤ 0.001	≤ 0.001	0.440	≤ 0.001
	6 meals schedule	≤ 0.001			

One-way ANOVA shows the significant daily variations of the clock-gene mRNA expressions in both treatment groups. Two-way ANOVA shows the significance of the difference between *ad libitum* and 6-meal schedule group.

No significant effects of *group* were detected, but a significant *interaction* effect was detected for two clock genes studied: Per2 and Dbp. Indeed, the levels of Per2 and Dbp transcripts displayed higher values in the scheduled regimen than in the *ad libitum* condition at ZT6.5 and ZT12.5, and ZT6.5 respectively ($p<0.05$).

Nonetheless, these data strongly suggest that the feeding schedule regimen, based on six meals delivered every four hours along the 24h L/D-cycle, does not disturb the 24h-rhythm of the five clock gene transcripts. Interestingly, under the same scheduled feeding conditions, it has been shown that the liver output (glucose rhythm) is not disrupted either [92].

Effect of hepatic sympathectomy (HSx) on plasma glucose rhythm under scheduled feeding conditions

VERIFICATION OF THE LIVER DENERVATION WITH PRV STAINING

Seven out of seventeen rats provided with jugular vein catheters were correctly denervated according to the virus patterns. No labeling was detected in the thoracal IML after 5-6 days. The data of the 10 HSx rats that did show labeling in the IML in the thoracic part of the spinal cord were not included in the final analysis of the HSx group.

LOCOMOTOR ACTIVITY AND GROWTH

Locomotor activity patterns measured in HSx rats (n = 4) were not significantly different from their activity before surgery (Figure 2). Initially some HSx rats had a delay in retaining their normal growth rate after the operation, but after a recovery period of

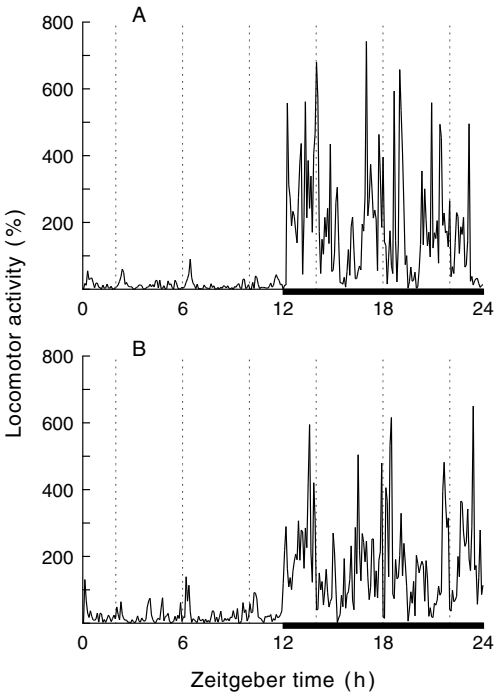

Figure 2 Locomotor activity levels (values are percentages of 24h-mean) across the light/dark cycle (A) in rats before surgery and (B) in the same rats after HSx under 6-meal schedule (n = 4). The dark bars indicate the dark period. The vertical dotted lines represent the 9- or 11-minutes periods when food was available.

10 days they had returned to their pre-operative body weight and consumed similar amounts of food as compared to the intact rats.

PLASMA GLUCOSE CONCENTRATIONS

During the experiment the catheter of one sham-operated animal got blocked and blood sampling was thus performed on seven sham-operated animals. The 24h profiles of plasma glucose concentrations (Figure 3) measured in the sham-operated rats showed a significant variation along the light/dark cycle ($p<0.001$) as also described previously [92, 100]. The highest and lowest concentrations were, respectively, ± 7.5mmol/l and ± 5.6mmol/l at ZT12.5 and ZT1.5, respectively. The data points of five sham-operated rats could be made to fit the single cosinor analysis, indicating that the plasma glucose concentrations displayed a 24h-rhythm, with a peak occurring at ZT 11.7 ± 0.8 (Goodness of fit (R^2) = 0.29 ± 0.10).

Plasma glucose concentrations of the seven well-denervated animals (HSx) also displayed a significant variation along the L/D cycle (Figure 3) ($p = 0.003$). The highest and lowest concentrations were, respectively, ± 7.5mmol/l and ± 6.1mmol/l at ZT6.5

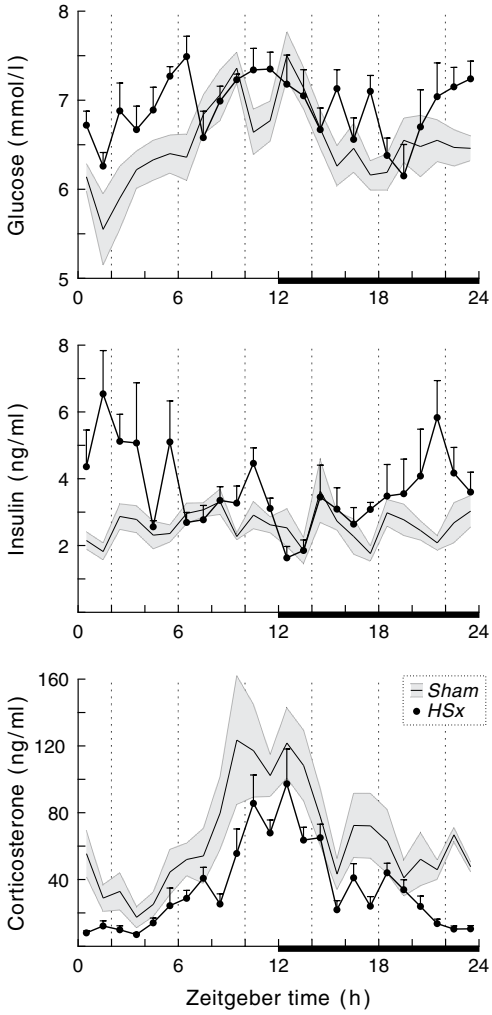

Figure 3 Plasma glucose, plasma insulin and plasma corticosterone concentrations across the light/dark cycle under scheduled feeding conditions in HSx (n=7) and sham-operated rats (n=7). The gray areas indicate the mean ± S.E.M plasma glucose/insulin/corticosterone concentrations in sham-operated animals. The black bars indicate the dark period. The vertical dotted lines represent the 9- or 11-minutes periods of food availability.

and ZT19.5. However, significant effects of *group* and *group*time* (Table 3) were detected by repeated-measures ANOVA, showing that the plasma glucose concentration in HSx differed significantly from the sham-operated group. In fact, the data from five out of the seven HSx animals could not be made to fit a cosinor analysis. The data points of the two HSx rat that did fit a cosinor analysis, however, presented glucose peaks at very different time points (ZT5 and ZT9). Accordingly, HSx rats did not show

Table 3 Statistical analysis of the diurnal variations of plasma glucose, plasma insulin and plasma corticosterone levels in sham-operated and HSx animals.

			Two-way ANOVA		
		Single	**Group (G)**	**Time (T)**	**Interaction (G x T)**
Glucose	Sham-operated	≤ 0.001	= 0.020	≤ 0.001	= 0.006
	HSx	= 0.003			
Insulin	Sham-operated	≤ 0.001	= 0.078	≤ 0.001	≤ 0.001
	HSx	≤ 0.001			
Corticosterone	Sham-operated	≤ 0.001	= 0.015	≤ 0.001	= 0.527
	HSx	≤ 0.001			

One-way ANOVA shows the significant daily variations of the plasma glucose, plasma insulin and plasma corticosterone concentrations in both groups. Two-way ANOVA shows the significance of the difference between sham-operated and HSx animals.

a 24h-mean rhythm in their plasma glucose levels as found for the sham-operated rats (Figure 3). Furthermore, the 24h-mean plasma glucose concentrations of HSx rats was slightly, but significantly, higher than the one of the sham-operated rats (6.92 ± 0.07 mmol/l vs 6.52 ± 0.13 mmol/l).

PLASMA INSULIN CONCENTRATIONS

The basal insulin concentrations in sham-operated animals (Figure 3) showed a clear variation over the light/dark cycle ($p<0.001$), with clear increments at every meal, as also described previously [92, 101]. However, only two out of the seven sham-operated animals presented a significant fit with a cosinor analysis with a peak at ZT7.2 ± 1.44 and an R^2 of 0.24 ± 0.07. Plasma insulin concentrations in HSx rats also displayed significant fluctuations over the L/D-cycle, but these were not clearly connected to the meals. No effect of *group* (Table 3) was detected, but repeated-measure ANOVA analysis did show a *group*time* effect, indicating that the 24h pattern of insulin in the HSx group differed significantly from that of the control group. In fact, in HSx rats more insulin profiles could be made to fit a cosinor (four out of seven), indicating a 24h-rhythm in plasma insulin concentrations, with peak concentrations at the beginning of the light period (ZT 0.93 ± 1.08) and an R^2 of 0.35 ± 0.05. Furthermore, the 24h-mean plasma insulin concentrations of HSx rats were higher than those of sham-operated rats (3.7 ± 0.4 mmol/l vs 2.6 ± 0.3 mmol/l).

PLASMA CORTICOSTERONE CONCENTRATIONS

The 24h profiles of plasma corticosterone concentrations (Figure 3) measured in the sham-operated rats showed a significant variation along the light/dark cycle ($p<0.001$), as previously described [92, 100]. No effect of g*roup*time* was detected by repeated-measure ANOVA (Table 3), indicating that the HSx rats displayed a similar 24h-rhythm in plasma corticosterone concentrations. Clearly, the data from both sham-operated

and HSx rats could be made to fit the cosinor analysis, indicating a rhythmic pattern with a peak occurring at the beginning of the dark period (ZT 12.34 ± 0.35) and an R^2 of 0.44 ± 0.15. However, the significant *group* effect revealed that (Table 3) the absolute 24h mean of plasma corticosterone concentrations was somewhat lower in HSx rats compared to sham-operated ones (34.49 ± 3.4 ng/ml and 59.19± 7.5 ng/ml, respectively).

Effect of hepatic sympathetic denervation (HSx) on liver clock gene expression under scheduled feeding conditions

No significant differences in rhythmicity in clock gene expression were found between non-operated and sham-operated rats ($p>0.05$), showing that the surgery *per se* did not affect the mRNA expression of the genes studied. Therefore, these two groups were combined into one control group.

The average NA concentration in the liver, measured in intact rats (n = 24), was 22.2 ± 1.7 ng/g of liver. In the HSx groups, two animals did not recover after surgery. The average of the NA concentration in the liver measured in the remaining twenty-eight HSx rats was 6.0 ± 2.2 ng / g of liver (i.e., ~ 30% of control levels). Thirteen out of these twenty-eight HSx rats displayed no detectable NA content and four HSx rats had <3.5 ng/g of liver (i.e., <15% of control levels). These four rats were included in the complete HSx group to increase the number of animals per time point and to allow statistical analysis. One-way ANOVA was performed on the data obtained from

Table 4 Statistical analysis of the daily expression of the clock gene mRNA in control and HSx groups.

			Two-way ANOVA		
		Single	**Time (T)**	**Group (G)**	**Interaction (G x T)**
Per1	Control	≤ 0.001	≤ 0.001	= 0.399	= 0.707
	Complete HSx	≤ 0.001			
Per2	Control	≤ 0.001	≤ 0.001	= 0.768	= 0.002
	Complete HSx	= 0.003			
Per3	Control	≤ 0.001	≤ 0.001	= 0.569	= 0.031
	Complete HSx	≤ 0.001			
Cry1	Control	≤ 0.001	≤ 0.001	= 0.925	≤ 0.001
	Complete HSx	= 0.016			
Dbp	Control	≤ 0.001	≤ 0.001	= 0.125	≤ 0.001
	Complete HSx	= 0.003			

One-way ANOVA shows the significant daily variations of the clock gene mRNA expression for each group. Two-way ANOVA shows the significance of the difference between control and HSx groups.

these seventeen HSx animals to estimate the impact of the sympathectomy on the rhythmicity of clock gene transcript levels (Table 4). All clock gene mRNA expression patterns in HSx rats show a rhythmic pattern over the L/D-cycle. Two-way ANOVA detected no significant effect of *group* for any of the clock genes, indicating that the feeding regimen plus the hepatic denervation did not impair the rhythmic pattern of the clock gene transcript levels (Figure 4). However, a significant effect of *group*time* (except for Per1) was detected, showing that hepatic sympathectomy plus feeding regi-

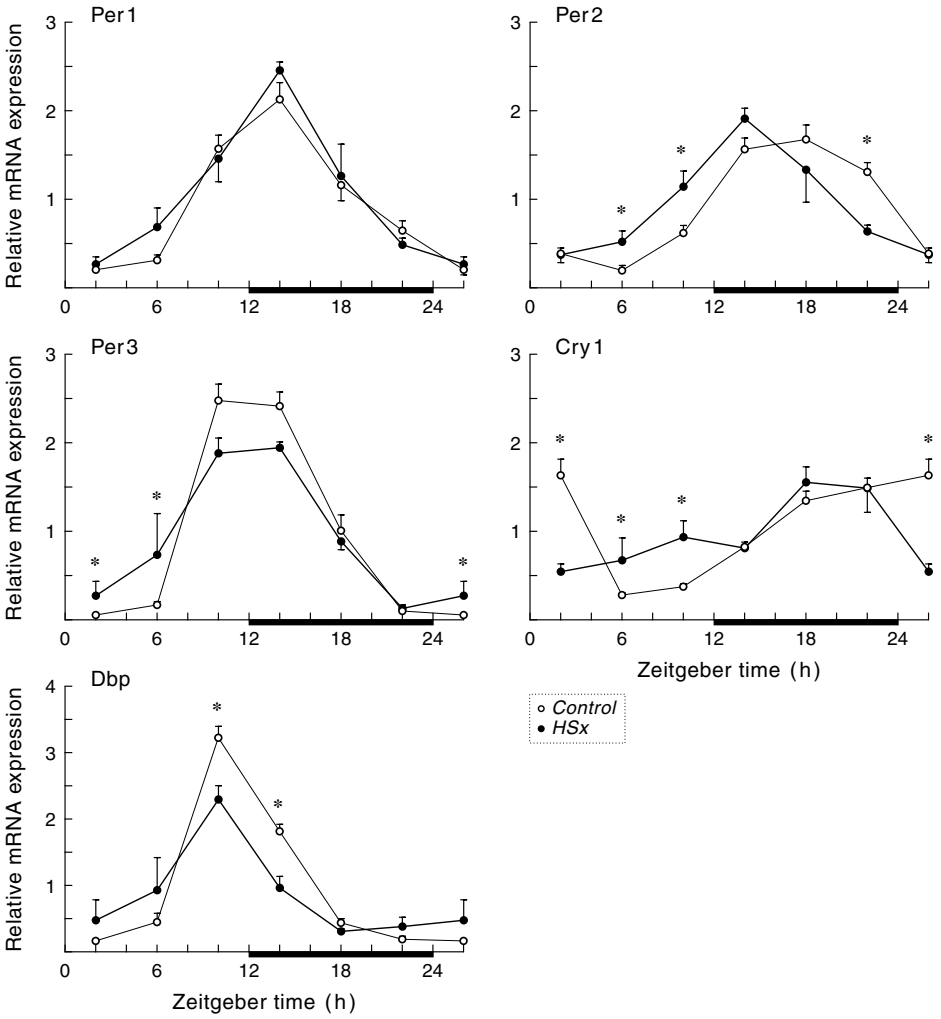

Figure 4: Daily rhythm in Per1, Per2, Per3, Cry1 and Dbp mRNA levels in the liver in HSx (n = 3, except at ZT14 n = 2) and control (n = 10) groups. The black bars indicate the dark period. In both groups, mRNA transcript levels displayed a rhythmic variation across the L/D-cycle (ANOVA, $p<0.05$). *, $p<0.05$ compared to the control group.

men does induce changes in the 24h profile of the clock gene mRNA expression. A 4h phase-advance of the Per2 peak was observed in HSx rats on the six-meal schedule, compared with the control group. This phase-advance of Per2 mRNA appeared not to be dependent on the hepatic denervation, since a phase-advance was also observed in the first experiment in intact rats placed under a scheduled regimen compared to *ad libitum*.

A similar shift seems to occur for the Cry1 mRNA levels in HSx rats compared with the control group. This effect seems to be induced by the hepatic sympathectomy, since a similar phase-shift was not detected in the first experiment. For Dbp and Per3, mRNA expression in HSx rats showed a peak with lower amplitude, suggesting that hepatic sympathectomy leads to a decline of the clock gene oscillation in the liver. In fact, Per2, Cry1 and Dbp displayed a slight increase of their p values in HSx group (0.003, 0.016, and 0.003 for Per2, Cry1 and Dbp, respectively), also indicative of a weaker oscillation in sympathectomized liver. In summary, clock gene transcript levels appear to be affected by the combined effects of scheduled feeding and hepatic denervation: a decrease of the peak amplitude (Per3, Dbp) and a phase-advance of the peak (Cry1, Per2). However, in both conditions the rhythmicity of the clock gene mRNA expression still remains. We also performed statistical analysis on a set of animals with an NA content below 50% (n = 23). This did not change the results of the statistical analysis.

Discussion

While the viral retrograde tracing technique showed that the SCN is connected to the liver via the sympathetic branch of the autonomic nervous system [154], the present study provides clear evidence that the contribution of the hepatic sympathetic branch is essential for the generation of the 24h-rhythm of plasma glucose. Activation of this sympathetic pathway causes an increase of plasma glucose concentrations [73], most probably via increased hepatic glucose production [202]. This multisynaptic SCN-liver connection thus provided us with the tool to investigate whether liver clock genes are involved in driving the rhythmic plasma glucose concentrations. Hereto we first submitted animals to a regular feeding schedule of six meals per day, to remove the (rhythmic) nocturnal feeding signal. With this non-circadian feeding pattern we demonstrated not only that the daily glucose rhythm was maintained in the blood, but also that the rhythmic pattern of clock gene expression was maintained in the liver, which showed that the daily feeding rhythm *per se* is not responsible for the rhythmicity in liver genes and plasma glucose. We then showed that disruption of the sympathetic input to the liver abolishes the rhythmicity of plasma glucose concentrations. However, in spite of the loss of this rhythmic liver output, the rhythmic expression of the clock-gene mRNA in the liver was not eliminated.

Neuronal and hormonal regulation of the glucose rhythm

Until now, many studies have shown a stimulatory effect of the sympathetic nerve on hepatic glucose production. *In vitro*, a stimulation of the sympathetic nerve in perfused liver induces a release of glucose [208]. Furthermore, stimulation of the SCN or PVN results in hyperglycemia, which is prevented by sympathetic blockers or denervation [73, 209]. Surprisingly, in our experiment, hepatic sympathectomy resulted in slightly elevated glucose concentrations along the light/dark cycle. This result, seemingly contradictory to previous studies, may be explained by the fact that we investigated the effect of disrupted sympathetic innervation on the plasma glucose concentrations only two weeks after the denervation and along a complete 24h L/D cycle, instead of during an acute stimulation experiment. An explanation for the elevated 24h glucose levels could be that the hepatic denervation of the sympathetic branch also includes sensory afferents. The disturbed feedback signal from the liver to the brain might then result in a plasma glucose concentration close to the daily peak level (7mmol/l). Indeed, glucose production is not only controlled by endocrine and neural factors but also by regulatory processes taking place within the liver itself. This so called auto-regulation process enables the control of hepatic glucose output, independent of changes in glucoregulatory hormones, and is mainly regulated by prevailing glucose concentrations [210].

HSx rats displayed a 24h-pattern in plasma insulin concentrations, with a peak at the beginning of the light period, whereas the daily rhythm in plasma glucose levels was eliminated, confirming that insulin alone is not sufficient to build the 24h-rhythm of glucose in the intact animal. Furthermore, the intact 24h-profile of corticosterone in HSx rats, which is similar to the profile of sham-operated rats, clearly shows that this hormone is too not sufficient for sustaining the daily variation of the plasma glucose levels, as also demonstrated previously [73].

All these observations together suggest that it is the removal of sympathetic input to the liver that is responsible for disrupting the daily variation of plasma glucose concentrations. This outcome of our experiments is in agreement with the study of Kalsbeek et al. (2004), which showed that the removal of the inhibitory input from the SCN to the PVN, by local infusions of TTX or a GABA antagonist, triggers an increase of plasma glucose concentration (most likely via an increase of hepatic glucose production [202]), which is prevented by a specific sympathetic denervation of the liver. In both studies we measured the plasma glucose concentration, which is not a direct measure of hepatic glucose production but the resultant of changes in hepatic glucose production, glucose influx from the intestines, and glucose uptake in tissues. For a definitive confirmation of our hypothesis, the SCN-PVN control on hepatic glucose production has to be investigated further by means of euglycemic clamp studies.

Neural and hormonal synchronization of the liver oscillator

Restricted feeding experiments have shown that the liver oscillator can be driven independently of the SCN in vivo [168, 171]. In this condition, uncoupling the L/D signal from the feeding activity leads to a shift of clock-gene transcription, in such a way that the liver transcript rhythms were out of phase with the master clock and locomotor activity. Interestingly, in our study we show that a regular feeding schedule does not disturb the rhythmic pattern of clock gene mRNA expression in the liver. However, our results are not contradictory to what has been shown previously. In our experimental model, the meals are distributed equally along the L/D cycle, which then leads to an a-rhythmic signal and thus to a removal of this part of the SCN output as putatively responsible for driving the liver oscillator. On the contrary, in the restricted feeding model the temporal food availability provides a strong and rhythmic signal, making food intake the most important Zeitgeber.

Plasma insulin and glucagon concentrations display a peak at each meal-time [92, 100, 101]. Previously, it has been suggested that insulin may act as a humoral signal on the liver oscillator [211, 212]. In our six-meal feeding schedule insulin and glucagon do not provide a timing signal to the liver to reset or extinguish its clockwork, since the a-rhythmic secretion does not disrupt the rhythmic pattern of the clock gene transcript in the liver.

Terazono *et al.* (2003) showed that daily injections of noradrenaline (NA) in SCN-lesioned animals generate Per mRNA oscillations in the liver, suggesting an adrenergic regulation of the clock gene oscillation in peripheral tissues. In our study, the disruption of the sympathetic input to the liver does not eliminate the diurnal pattern of the clock-gene transcripts, indicating that sympathetic innervation is not necessary for maintaining the rhythmic clock gene expression within the liver. This discrepancy is not surprising at all. In fact, chronic injection of NA at fixed times during 6 days in an SCN-lesioned rat provides the liver (and the rest of the body) with a rhythmic signal, such as restricted feeding, which turns NA into the predominant Zeitgeber for the peripheral oscillator. In our model, HSx rats still display rhythmic locomotor activity (in phase with the L/D cycle) and a 24h-rhythm in plasma corticosterone - two components suggested to play a role in the transmission of SCN signals to the peripheral clock [168, 179, 181]. Thus, even after disruption of the sympathetic innervation and removal of the rhythmic feeding signal, the SCN is still able to provide its timing signal to the liver oscillator, via hormonal, neuronal (parasympathetic branch), behavioral or other cues.

In our experiment we observed that, despite a combined absence of rhythmic feeding and sympathetic innervation, the clock gene rhythm is still maintained, although sometimes with a weakened oscillation. Clearly the present results show that removal of the sympathetic input together with a scheduled feeding regimen, leads to a disrupted plasma glucose rhythm, but is not enough to eliminate the daily rhythmicity of clock genes within a tissue. Consequently, the remainder of the endocrine, behav-

ioral and neuronal outputs of the SCN may compensate for this loss. However, the maintained oscillation in the liver observed in HSx two weeks after surgery, could also be explained by the fact that the liver contains a self-sustained oscillator [200, 213]. In conclusion, as suggested before, under normal physiological conditions the liver may integrate endocrine (e.g. corticosterone), neuronal (sympathetic and parasympathetic branch) and behavioral (locomotor activity; body temperature) outputs from the SCN in order to maintain or synchronize clock gene rhythm [178]. Furthermore, we demonstrated that the loss of the 24h-rhythm in plasma glucose levels induced by hepatic sympathectomy is not correlated to an impairment of the clockwork of the liver oscillator. Although the sympathetic denervation did not directly affect the liver clockwork *per se*, it could still interfere with the downstream rhythmic signalization leading then to an a-rhythmic activity of enzymes involved in glucose metabolism. This would imply that clock gene rhythmicity is not directly connected to the rhythmic activity of those enzymes. Indeed, it has recently been shown that the parasympathetic input to the liver may act on the hepatic mRNA expression of enzymes involved in glucose metabolism [214]. Our results also fit in nicely with recent reports indicating an important role for the vagal input to the liver in the control of the central nervous system over hepatic glucose production. The hepatic branch of the vagus nerve was shown to be essential for transmitting the inhibitory effect of hypothalamic insulin and free fatty acids on the hepatic glucose production [214, 215]. Together these data indicate an important role for the autonomic nervous innervation of the liver as a mediator of the hypothalamic control on the hepatic glucose production and plasma glucose concentrations.

In summary, these data provide evidence that the SCN needs the sympathetic branch to generate a 24h-rhythm in plasma glucose concentrations, independently of hormonal changes (e.g. insulin, corticosterone). In addition, this autonomic branch is not necessary to sustain and/or reset the liver oscillator. In contrast to studies performed on cell division in liver regeneration [216] we did not find any correlation between rhythmic output (e.g. glucose) and rhythmicity of clock gene transcript levels. Further studies investigating the activity of the liver enzymes involved in gluconeogenesis and glycolysis are required to elucidate the exact mechanism responsible for the loss of rhythmicity of glucose concentrations in denervated animals.

Acknowledgments

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Chapter 3

Daily rhythms in metabolic liver enzymes and plasma glucose require a balance in the autonomic output to the liver

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Submitted

Abstract

Daily variations in plasma glucose concentrations are controlled by the biological clock, located in the suprachiasmatic nucleus (SCN). Our previous studies indicated an important role of the sympathetic innervation of the liver, but not of local clock genes, in the generation of the daily glucose rhythm. Here we show that autonomic innervation of the liver, but not SCN-driven food intake, contributes to the circadian regulation of hepatic glucose metabolism and plasma glucose levels. However, even a-rhythmic feeding combined with a complete denervation of the autonomic input to the liver did not abolish the 24h-profile in plasma glucose completely, nor did it abolish all rhythms in liver enzymes. These results show that the SCN uses multiple mechanisms to control rhythmic hepatic glucose output. Interestingly, contrary to complete denervation, selective sympathetic or parasympathetic denervation of the liver does disturb both glucose and liver enzyme rhythmicity, demonstrating that an unbalanced autonomic output to the liver has a deleterious effect on the maintenance of rhythmic liver metabolism.

Introduction

One of the main features of homeostasis is the capacity to predict upcoming changes in the environment allowing the organism to anticipate and adapt to those changes. As such the organism anticipates changes in the season and light/dark cycle, resulting in increases in food intake and fat storage just before winter, while anticipation to the daily activity period results in an increase in blood glucose levels just before the activity begins [201].

Since glucose is the essential fuel for the brain, glucose metabolism must be tightly regulated in order to maintain plasma glucose levels within narrow limits, and the brain is thus intimately involved in organizing blood glucose levels, not only within an optimal physiological range but also according to the time of the day, in which the biological clock or the suprachiasmatic nucleus (SCN) plays a major role [65, 71]. In fact, the daily variation in plasma glucose concentrations, characterized by a peak before

the onset of the activity period, is driven by the SCN, independent of the daily rhythm in feeding activity [92]. A physiologically quite meaningful fact is that the highest plasma glucose concentrations and glucose tolerance coincide just before awakening, indicating that an increase of glucose output by hepatic glucose production is required to compensate for the high glucose uptake [73, 89]. The sympathetic input to the liver plays an essential role in the daily rise in plasma glucose [73, 217]. In accordance with these observations is that, within the liver, key enzymes involved in glucose metabolism display an oscillatory pattern in their gene expression along the L/D cycle, confirming the potential role of the SCN in the circadian pattern of hepatic gene expression [161, 218, 219]. Since we observed blood glucose a-rhythmicity but no disturbance in liver clock gene rhythms after sympathetic liver denervation [217], we decided to investigate, under the same experimental conditions (i.e., regular feeding combined with sympathetic denervation of the liver), to what extent the abolishment of the glucose rhythm could be attributed to a loss of circadian expression in metabolic enzymes, involved in hepatic glucose metabolism (e.g., Phosphoenolpyruvate carboxykinase (*PEPCK*), Glucose-6-Phosphatase (*G-6-Pase*), Glucokinase (*GK*)). The outcome of this study indeed demonstrated a disturbed metabolic enzyme expression after hepatic sympathectomy, suggesting a-rhythmic glucose output. Since the two branches (sympathetic and parasympathetic) of the ANS can influence hepatic glucose metabolism by controlling the hepatic glucose production [73, 153, 220], we decided to investigate the separate effects of the ANS and rhythmic food intake on the circadian expression of the metabolic gene expression by performing a complete (or sham) denervation of the liver in animals subjected to rhythmic or a-rhythmic feeding conditions, i.e., *ad libitum* or 6-meal schedule, respectively.

Materials & Methods

Animals

Adult male Wistar rats (Wu, Harlan, Zeist, The Netherlands) were kept in a 12 h light/12 h dark cycle (L/D; lights on at 7:00 Zeitgeber time (ZT) 0) under constant conditions of temperature (20 ± 2°C) and humidity (60 ± 2%) with water and food *ad libitum* (Global Rodent Breeding, purified pellet, Harlan Teklad). All experiments were conducted with the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Surgical procedure

Surgery was performed on rats weighing 300-350g. A mixture of Hypnorm (Janssen, 0.05ml/100g BW, i.m.) and Dormicum (Roche Nederland, 0.04ml/ 100g BW, s.c.) was used to anesthetize the animals. After closing the wound with atraumic sutures (5-0 Perma-Hand Said Ethicon), the animals were injected subcutaneously with saline to

prevent dehydration. Post-operative care was provided by subcutaneous injection of 0.01ml/100g BW of pain killer (Temgesic, Schering-Plough (Utrecht importer)) after the operation and on the following day.

HEPATIC PARASYMPATHECTOMY

The fascia containing the hepatic branch was stretched by gently moving the stomach and the oesophagus. With a myelin-specific dye (Toluidin Blue) the hepatic branch was revealed as it separated from the left vagal trunk. The stained neural tissue was transected between the ventral vagus trunk and the liver. Also, small blue-stained branches running in the fascia between the stomach and the liver were transected. The denervation procedure was performed as previously described [73, 221].

HEPATIC SYMPATHECTOMY

The liver lobes were gently pushed up in order to be able to access the bile duct and the portal vein complex. At the level of the hepatic portal vein the hepatic artery divides into the hepatic artery proper and the gastroduodenal artery. At the point where the hepatic artery divided into the hepatic artery proper and the gastroduodena artery, the arteries were separated from the portal vein via blunt dissection. The nerve bundles running along the hepatic artery proper were visualized using a myelin-specific dye (Toluidin Blue) and then transected [73, 217]. At the end of the experiment, the completeness of the sympathetic denervation was checked by HPLC measurement of hepatic noradrenalin (NA) content; animals with a non-complete denervation were systematically removed from the data analysis.

JUGULAR VEIN CATHETER

During the same surgical procedure (see above), an intra-atrial silicone cannula was implanted through the jugular vein according to the method of Steffens [203] for all the groups whose glucose, insulin and corticosterone had been measured.

Experimental Set-up

EXPERIMENT 1 – EFFECT OF A SYMPATHETIC LIVER DENERVATION ON METABOLIC GENE EXPRESSION

It has been shown previously that scheduled feeding combined with hepatic sympathectomy impairs the daily rhythm of plasma glucose concentrations [217]. In the first part of this study we checked whether, under the same experimental conditions, the loss of the glucose rhythm could be correlated with an abnormal daily expression profile of metabolic enzymes in the liver. To this end, we analyzed the daily profile of metabolic enzyme expression (*G-6-Pase*, *GK*, Pyruvate kinase (*PY*), Glucose transporter 2 (*GLUT2*), *PEPCK*) and the glycogen content in the liver along the Light/Dark cycle in a group of sham-operated animals placed under *ad-libitum* conditions (n= 5 per time point) and a group of HSx rats (n = 3 per point except ZT14 = 2) subjected to a 6-meal schedule.

EXPERIMENT 2 - COMPLETE HEPATIC DENERVATION IN ANIMALS UNDER AD LIBITUM CONDITIONS

Effect on the daily profile of plasma glucose/corticosterone and insulin concentrations Rats were divided into two groups: sham-operated (n = 10) or a complete denervated (CD) group (n = 14). Two weeks after surgery, 0.2ml of blood was collected, for the measurement of plasma glucose, insulin and corticosterone concentrations, once every hour for 12 consecutive hours on two different occasions within a period of two weeks. The two runs started at ZT6.5 and ZT18.5. Blood samples were kept in – 80°C until analysis.

Effect on metabolic enzyme expressions/ Glycogen content Rats were divided into two groups: sham-operated (n = 30) and one CD group (n = 42). After two post-operative weeks, groups of animals were sacrificed every four hours along the L/D-cycle (ZT2, 6, 10, 14, 18 and ZT22).

EXPERIMENT 3 - COMPLETE HEPATIC DENERVATION IN ANIMALS SUBJECTED TO A SCHEDULED FEEDING REGIMEN

For this experiment, rats were entrained to the scheduled feeding regimen for three weeks and they were considered to be adapted to the feeding schedule when they ate ± 3g at every meal. This regular feeding was maintained until the end of the experiments. For more details on the scheduled feeding regimen, see [217].

Effect on the daily profile of plasma glucose/corticosterone and insulin concentrations Rats were divided into two groups: sham-operated (n = 8) or CD (n = 12). Two weeks after surgery, 0.2ml of blood was collected once every hour (for measurement of plasma glucose, insulin and corticosterone concentrations) during 12 consecutive hours on two different occasions within a period of two weeks. Blood samples were kept in –80°C until analysis.

Effect on metabolic enzyme expression/ Glycogen content The CD group contained 30 rats, i.e., 5 animals per time point. After two post-operative weeks, animals were sacrificed every four hours along the L/D-cycle (ZT2, 6, 10, 14, 18 and ZT22).

End of the experiments

Animals were sacrificed by decapitation. Liver pieces were collected and directly frozen into nitrogen and kept at –80°C until analysis.

High Performance Liquid Chromatography-Electrochemical Measurements

Since the sympathetic denervation we performed earlier, in contrast to the parasympathetic one (i.e., section of the nerve stalk), had not been a 100% of success [217], we checked the completeness of the sympathectomy. Pieces of liver were taken after experimental procedures and processed using High Performance Liquid Chromatography (HPLC) with electrochemical detection for noradrenaline (NA) content. Tissue samples were homogenized in ice-cold 0.1M perchloric acid (2ml/100mg) and centri-

fuged twice (14000 rpm) for 15 minutes at 4°C. Samples of 20µl supernatant were subsequently transferred to the HPLC for analysis (Waters 600E pump and Waters 717plus autosampler, Waters Chromatography b.v., Netherlands; Decade VT-03 electrochemical detector, Antec Leiden, Netherlands and Shimadzu Class-vp ™ software v.5.03 Shimadzu Duisburg, Germany). The mobile phase consisted of 0.06mol/l Natrium Acetate, 9mmol/l Citric acid, 0.37mmol/l Heptasulfonic acid and 12.5% methanol. The flow rate was kept constant at 0.3ml/min. Separation of NA from other endogenous compounds was achieved with a Supelcosil column (LC-18-DB 25cm x 4,6mm x 5 µm), with a 2 cm guard-column of the same material (Supelco Superguard ™, Supelco USA). Column temperature was kept constant at 28°C. Quantification was achieved by means of electrochemical detection (+0.65V) and NA content was measured against a calibration curve of an external NA standard. The detection limit (ratio signal to noise = 2) was 1 pg of NA injected on column. Sympathetic denervation is considered to be complete when the NA content is below 10% of that of intact-liver animals [222].

Measurement of the hepatic glycogen content

Frozen liver tissue was crushed on dry ice and then the liver powder was homogenized into 0.1 M potassium hydroxide (KOH) at pH= 13. Homogenization tubes were placed in a water bath (80°C) for 30 min, and then placed on ice and left to reach room temperature. Formic Acid was added to the homogenized solution to adjust the pH to 4, and then centrifuged for 1 min at 14 000rpm. The aliquots were collected in new Eppendorf™ tubes. For each sample, 100ul was incubated with 10ul of Amylo-α-1.4-α1,6-glucosidase (from stock solution diluted ½, cat. 102857, Roche; Nederlands) at 40°C for 2 hours [223-225]. By way of control, i.e. "free" glucose levels in the liver, 100ul of the sample was incubated without the amyloglucosidase for 2 hours. As a standard, replicate rabbit liver standard (Sigma, G-8876, Nederlands) was used and treated in exactly the same way as the samples. Glucose concentrations were determined in the supernatant, using the Glucose/GOD-Perid method (Boehringer Mannheim, GmGH. Germany). The glycogen content in the liver tissue was calculated by (glucose concentration measured in the sample – the control one) and converted to glycogen pg/mg of tissue by using the replicate rabbit liver standard.

Plasma measurements

Plasma glucose concentrations were determined in triplicate using the Glucose/GOD-Perid method (Boehringer Mannheim, GmGH. Germany). Plasma immunoreactive insulin concentrations were determined in duplicate with a radio immuno-assay kit (Linco Research, St. Charles, MO). The lower limit of the assay was 0.2ng/ml and the coefficient of variation of the immunoassay was less than 8 %. Plasma corticosterone concentrations were determined in duplicate with a radio-immuno-assay kit (ICN

Biomedicals, Costa Mesa, CA). From the samples, 10µl was taken and diluted in 4 ml of assay buffer. The lower limit of the assay was 1ng/ml and the coefficient of variation of the immunoassay was less than 4 %.

Rat liver RNA extraction and cDNA synthesis

Total RNA was extracted from each liver collected at the different ZT time points by RNeasy mini Kit (Qiagen, Courtaboeuf, France) and included DNase step according to the manufacturer's instructions. For the cDNA synthesis, 2µg of total RNA was reverse-transcribed with Superscript II RNase H^- Reverse transcriptase (SSIIRT, In vitrogen, Life Technology Nederland) according to the manufacturer's instructions. As a control, 2 µg total RNA followed the same procedure, except that 1µl of SSIIRT was replaced by 1µl of water (No Reverse-Transcriptase or No RT). Each single stranded cDNA and their respective No RT were then used as a template for the Real-Time PCR.

Real-Time PCR (RT-PCR)

Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primer data are given in Table 1. mRNA expression of enzyme involved in glucose metabolism [Glucose 6 phosphatase (*G6Pase*), Glucokinase (*GK*), Glucose transporter 2 (*GLUT2*), Phosphoenolpyruvate Carboxykinase (*PEPCK*)] and two reference genes [(ubiquitin conjugate enzyme (*Ubc2e*) and TATA box binding protein (*Tbp*)] were analyzed by RT-PCR .

One µl of each cDNA was incubated in a final volume of 20µl RT-PCR reaction containing 1X SYBR-green master mix (Applied Biosystems) and 6 pmol (reverse + forward) of each primer. Quantitative PCR was performed in an Applied Biosystems (Model ABI7300 Prism Sequence Detection System). The amount of each transcript was first normalized independently, with the average calculated from the transcript levels along the L/D as previously described [217].

Table 1: Gene nomenclature, GenBank accession code, primer sequences and primer efficiency.

Gene	GenBank	Forward primer	Reverse primer	Efficiency
G6Pase	NM 013098	CCCATCTGGTTCCACATTCAA	GGCGCTGTCCAAAAAGAATC	1.95
GK	NM 012565	TCCTCCTCAATTGGACCAAGG	TGCCACCACATCCATCTCAA	1.98
GLUT2	NM 012879	GAAGGATCAAAGCCATGTTGG	CCTGATACGCTTCTTCCAGCA	1.98
PY	NM 012624	GAGAGTTTTGCAACCTCCCCA	CCTTCACAATTTCCACCTCCG	1.95
PEPCK	NM 198780	TGCCCTCTCCCCTTAAAAAAG	CGCTTCCGAAGGAGATGATCT	2.02
Tbp	XM 217785	TTCGTGCCAGAAATGCTGAA	TGCACACCATTTTCCCAGAAC	1.91
Ubi	U 13176	CTCCAACAGGACCTGCTGAAC	CTGAAGAGAATCCACAAGGAATTGA	1.8

Statistical analysis

GLUCOSE, CORTICOSTERONE AND INSULIN PROFILES

The plasma concentrations of glucose, corticosterone and insulin are expressed as mean ± SEM. Statistical analysis was conducted using a repeated-measures analysis of variance (ANOVA) to test for an effect of time. If ANOVA detected a significant effect of time, a cosinor analysis was performed, using the fundamental period (24h) for the individual sets of data points. Curve fitting was performed using constrained nonlinear regression analysis (with the SPSS Advanced statistic 11.0), and parameters whose significant level of the fitted curve was less than 0.05 were used for further calculation of parameter means and for examination of the modulation of the rhythm throughout the L/D cycle [204]. The fitted function is defined by its mesor (rhythm-adjusted mean), amplitude (50% of the difference between the maximum and the minimum), and acrophase (time of the maximum). ANOVA and the Student's t-test were used to detect significant differences between sham-operated and HSx or CD rats. For the ANOVA, (paired) t-tests and the cosinor analysis, $p<0.05$ was considered to be a significant difference. In all cases, statistics and cosine analysis were done on absolute values.

RT-PCR AND GLYCOGEN DATA ANALYSIS

Data from all experiments are presented as mean ± S.E.M. To demonstrate a statistically significant difference among the different ZT time-points, the P-value of One-way ANOVA was calculated on the basis of the normalized data (SPSS package V12.0). When the ANOVA analysis indicated such a difference, post hoc (paired) were performed with a significance set at 0.05. To evaluate an effect of groups (control / experimental groups), a two-way ANOVA was performed with a significance set at 0.05. The student t-test (un-paired) was applied to estimate a significant difference between two groups at a specific ZT point.

Results

The daily mRNA profile of liver metabolic enzymes and glycogen content

Under *ad libitum* conditions, only the daily profiles of *Glucokinase*, *PEPCK* mRNA expression and glycogen content (Figure 1) follow a rhythmic pattern along the L/D cycle ($p< 0.05$) in accordance with data previously described [166, 218, 219, 226, 227]. Glucokinase, the enzyme involved in glucose uptake by transforming glucose to glucose 6 phosphate in the liver, displayed a higher mRNA level during the night period, when the rats consume most of their food. *PEPCK*, the key enzyme for gluconeogenesis, shows an elevated expression at the end of the day (ZT10), when the hepatic glycogen content is low. Despite the fact that *G6Pase* gene expression did not show a significant effect of time ($p = 0.19$), we did observe a clear difference between the day and night mRNA levels as previously described [228]. The diurnal gene expression of *G6Pase* also fits with the daily profile of its enzymatic activity [229, 230]. Since G6Pase is involved in

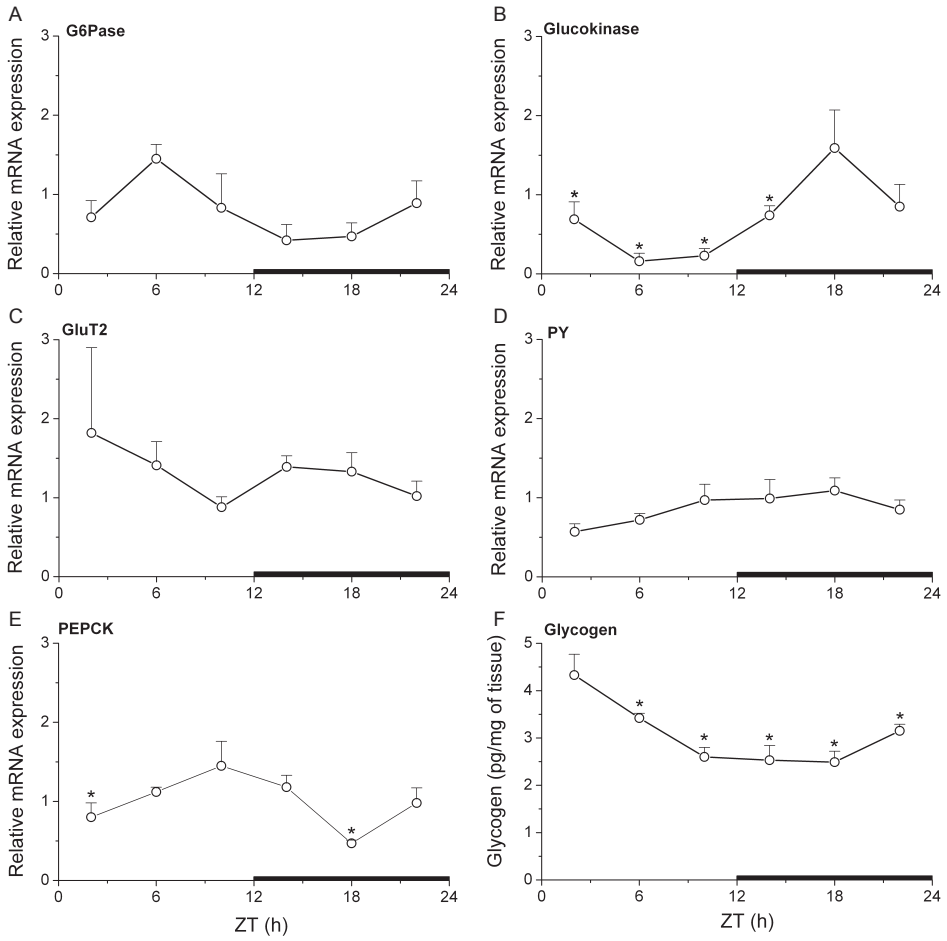

Figure 1 Daily profile in *G6Pase*, *GK*, *GLUT2*, PY and *PEPCK* mRNA levels and glycogen content in the liver of sham-operated rats (data are represented as the mean ± S.E.M). Animals were housed in 12h light/12h dark cycle with lights on at 07:00 under *ad libitum* conditions and were sacrificed (n = 5 for ZT10/18 and 22; n = 4 for ZT2/6 and 14) at the indicated times of the daily cycle (Zeitgeber Time (ZT)). The peak of mRNA expression for each liver enzyme was compared to that of other time points and was considered significantly different (*) for $p < 0.05$. The black bars indicate the dark period.

glucose output and *GK* in glucose uptake, it makes sense to observe the daily profile of their gene expression in an opposite phase [185]. No daily pattern in gene expression was observed for pyruvate kinase (*PY*), an enzyme of the glycolytic pathway, or for *GLUT2* which is involved in the release of glucose into the bloodstream (Table S1).

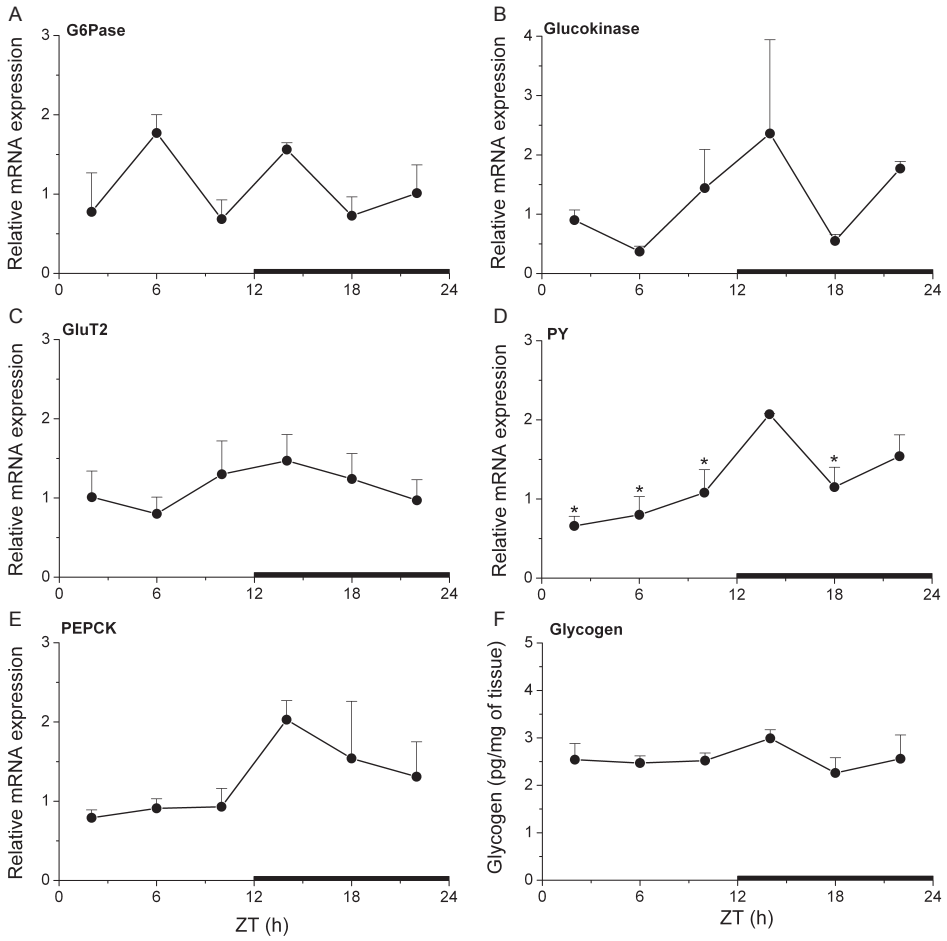

Figure 2 Daily profile in metabolic enzyme mRNA levels and glycogen content (data are represented as the mean ± S.E.M) in the liver of HSx animals subjected to a 6-meal schedule (n = 3 except for ZT14 n = 2). The peak of mRNA expression for each liver enzyme was compared to that of other time points and was considered significantly different (*) for $p < 0.05$.

Effect of sympathetic liver denervation on metabolic gene expression

The rhythmic profiles of all examined liver enzymes and liver glycogen content is affected in HSx rats subjected to scheduled feeding (Figure 2). Despite an increase in mRNA level for all enzymes at ZT14, only *PY* displayed a significant effect of time ($p = 0.02$). Plasma glucose levels after the removal of the sympathetic input to the liver do not show a daily rhythmic pattern (Figure S1). In addition to a disturbed rhythm in the expression of hepatic enzymes, the glycogen rhythm is also abolished and exhibits a low level. These disturbed daily patterns in liver enzyme expression

and hepatic glycogen content after HSx suggest an impairment of the circadian glucose metabolism within the liver, resulting in a loss of the rhythm in plasma glucose concentrations [217].

Complete hepatic denervation in ad libitum fed animals

In our previous study we showed that denervation of the sympathetic branch of the ANS resulted in a loss of the 24h-rhythm in plasma glucose levels. Moreover, we also observed that after denervation of the parasympathetic branch (HPx) of the liver the glucose rhythm in the blood is abolished (Figure S2), indicating an essential role of the ANS input to the liver in order to generate the glucose rhythm.

To examine the relation between circadian profile of the liver enzymes and rhythmic plasma glucose concentrations, we aimed to discriminate which liver enzymes are driven by the ANS and which by the feeding activity. Therefore we studied the influence of the autonomic input separate from that of food intake, and examined the 24h-profile of metabolic liver enzyme expression and glycogen content in completely denervated animals, combined with rhythmic and a-rhythmic food intake (i.e. ad libitum and regular feeding, respectively). As a first step, we investigated the effect of a complete denervation *per se* on liver metabolic components in relation with plasma glucose levels.

EFFECT ON THE DAILY PROFILE OF PLASMA GLUCOSE/CORTICOSTERONE AND INSULIN CONCENTRATIONS

Plasma glucose concentrations The 24h profile of plasma glucose concentrations (Figure 3A) measured under ad libitum conditions in the sham-operated rats showed a significant variation along the light/dark cycle ($p<0.001$) as also described previously [92, 100] with a peak occurring at ZT 11.3 ± 1.21 (Goodness of fit (R^2) = 0.37 ± 0.12). Plasma glucose concentrations of the 9 CD animals also displayed a significant variation along the L/D cycle (Figure 3A) ($p<0.001$), showing that the denervation did not affect the rhythmic profile of the plasma glucose levels. In fact, the data from the 9 CD animals could fit a cosinor analysis, indicating a day/night change in the plasma glucose levels. Significant effects of *group*time* were detected by repeated-measures ANOVA, showing that the daily pattern in plasma glucose concentrations in CD differed from the sham-operated group. Indeed, CD animals exhibit significantly higher plasma glucose concentrations during the daytime compared to those in sham-operated rats ($p = 0.02$). However, the 24h-mean plasma glucose concentrations of CD rats did not significantly differ from those of sham-operated rats (6.36 ± 0.13 mmol/l vs 6.90 ± 0.19 mmol/l).

Plasma corticosterone concentrations The 24h profiles of plasma corticosterone concentrations (Figure 3B) measured in the sham-operated rats showed a significant variation along the light/dark cycle ($p<0.001$), as previously described [92, 100]. One-way ANOVA with repeated measurement also indicates a significant variation in plasma corticosterone levels along the L/D cycle in CD rats ($p<0.001$). The data from both

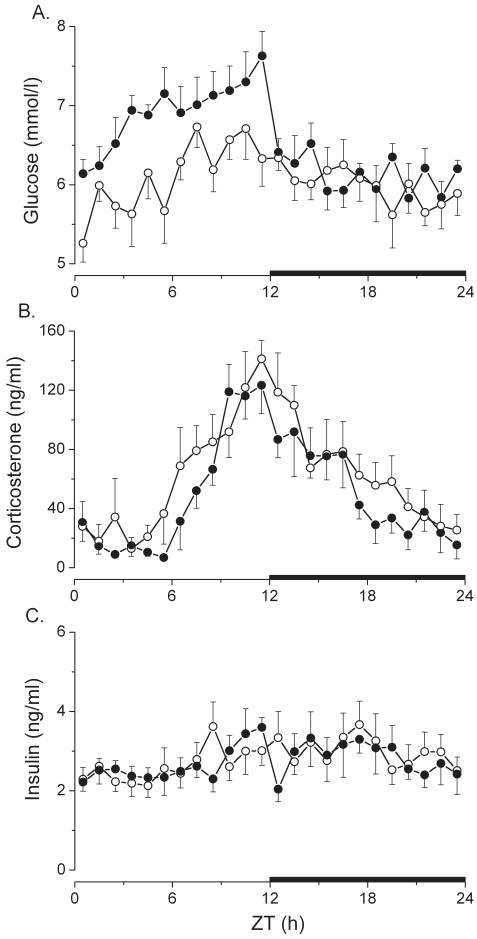

Figure 3 Plasma glucose, plasma insulin and plasma corticosterone concentrations across the light/dark cycle in CD (●; n = 8) and sham-operated rats (o; n = 9) under *ad libitum* conditions. The data are represented as the mean ± S.E.M. The black bars indicate the dark period.

sham-operated and CD rats could be fitted with the cosinor analysis: a peak was detected at ZT 12.12 ± 1 (R^2 of 0.56 ± 0.14) and ZT 12.19 ± 0.5 (R^2 of 0.53 ± 0.15) for sham and CD rats respectively, indicating that the CD rats displayed a similar 24h-rhythm in plasma corticosterone concentrations. The 24h-mean plasma corticosterone concentrations in CD rats showed lower mean levels as compared to those of sham-operated rats (50.23 ± 5.22 mmol/l vs 62.32 ± 5.68 mmol/l).

Plasma insulin concentrations In spite of the higher daytime glucose values the basal insulin concentrations in sham-operated and CD animals (Figure 3C) did not show a variation over the light/dark cycle (p=0.07 and p=0.21, respectively). Two way ANOVA

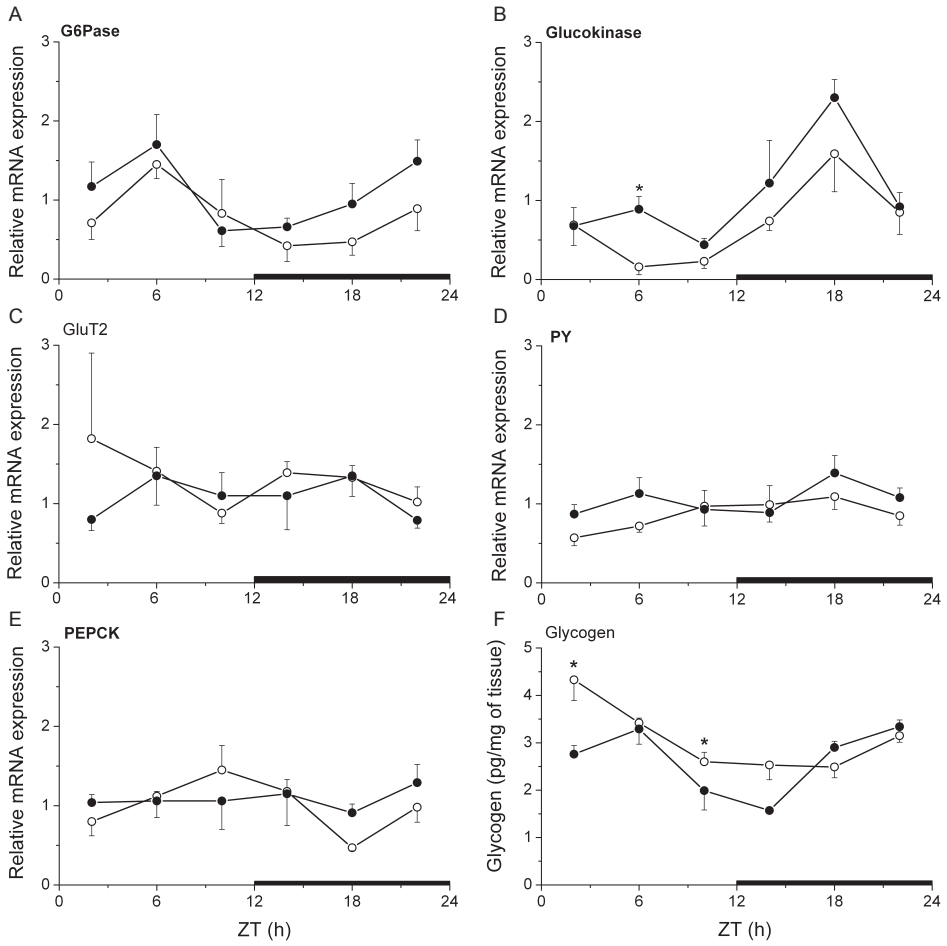

Figure 4 Daily profile in glucose-metabolizing enzyme and hepatic glycogen content in CD (●; n = 5 for ZT2/6, n = 4 for ZT= 10, n = 3 for ZT14/22 and n = 6 for ZT18) and sham-operated animals (o; see figure 1) fed *ad libitum*. The data are represented as the mean ± S.E.M. The black bars indicate the dark period. In both groups, some mRNA transcript levels displayed a rhythmic variation across the L/D-cycle (ANOVA, $p<0.05$). *, $p<0.05$ compared to the control group. For statistics see Table S1.

did not disclose an effect of *group* and *group*time*, indicating that the denervation *per se* does not affect the insulin pattern over the L/D cycle. Furthermore, the 24h-mean plasma insulin concentrations in CD rats did not significantly differ from those of sham-operated rats (2.74 ± 0.25 mmol/l vs 2.81 ± 0.31 mmol/l).

EFFECT ON METABOLIC ENZYME EXPRESSION/ GLYCOGEN CONTENT

Two-way ANOVA did not disclose a significant effect of *group* for any of the studied enzymes and glycogen content, showing that the complete denervation *per se* in CD

animals fed *ad libitum* does not affect the daily profile in the metabolic components in the liver (Figure 4). In contrast with our expectations, *GK* gene expression exhibits a rhythmic pattern with high expression at night and *G6Pase* mRNA displays a daily profile with an opposite phase just as in the sham operated animals after complete liver denervation. Moreover, the circadian profile in *GK* mRNA expression in CD animals exhibited a somewhat higher amplitude during the day time as compared to the sham-operated animals (*group*time* effect). Glycogen content in CD rats remained rhythmic ($p<0.001$), but a significant effect of *Group* and *group*time* was detected, showing that denervation does modify the 24h profile of the hepatic glycogen i.e., lower levels at the beginning of the light period (Table S1). Remarkably, only *PEPCK* mRNA expression did not show a rhythmic pattern in the CD group ($p = 0.9$), in contrast to the sham-operated one ($p = 0.02$), indicating that the ANS controls the rhythmic gene expression of *PEPCK*. The absence of a *group* or *group*time* effect may be explained by the intermediate levels (average between the high/lowest values) of *PEPCK* mRNA through the L/D.

The upregulation of *G6Pase* gene expression during the daytime (a fasting period) might be explained by a increase of hepatic glucose production (i.e., glycogenolysis and gluconeogenesis) in order to release glucose in the bloodstream, whereas at night its expression is inhibited by the food intake [231]. Glucokinase, an enzyme involved in hepatic glucose uptake, exhibits a diurnal variation in its mRNA expression in an opposite phase to the *G6Pase*, i.e., upregulation of gene expression when glucose supply is increased by food intake during the night period [232]. Consequently we hypothesized that the sustained-oscillatory mRNA expression in *GK*, *G6Pase* and glycogen in CD animals is driven by the rhythmic feeding activity, and we decided therefore to study blood glucose levels and liver metabolic enzyme expression after CD, independent of food intake.

Complete hepatic denervation in animals subjected to scheduled feeding regimen

CD animals were entrained to a-rhythmic feeding, i.e., 6 meals per 24 hours, a schedule to remove the SCN driven day/night rhythm in food intake.

EFFECT ON THE DAILY PROFILE OF PLASMA GLUCOSE/CORTICOSTERONE AND INSULIN CONCENTRATIONS

Plasma glucose concentrations The 24h profiles of plasma glucose concentrations (Figure 5A) measured in the sham-operated rats subjected to the 6-meal schedule showed a significant variation along the light/dark cycle ($p<0.001$) as also described previously [92, 100]. The data points of 5 out of the 6 sham-operated rats could be fitted to a single cosinor analysis, indicating that the plasma glucose concentrations displayed a 24h-rhythm, with a peak occurring at ZT 9.8 ± 0.62 (Goodness of fit (R^2) = 0.34 ± 0.14). Plasma glucose concentrations of the 7 CD animals also displayed a significant

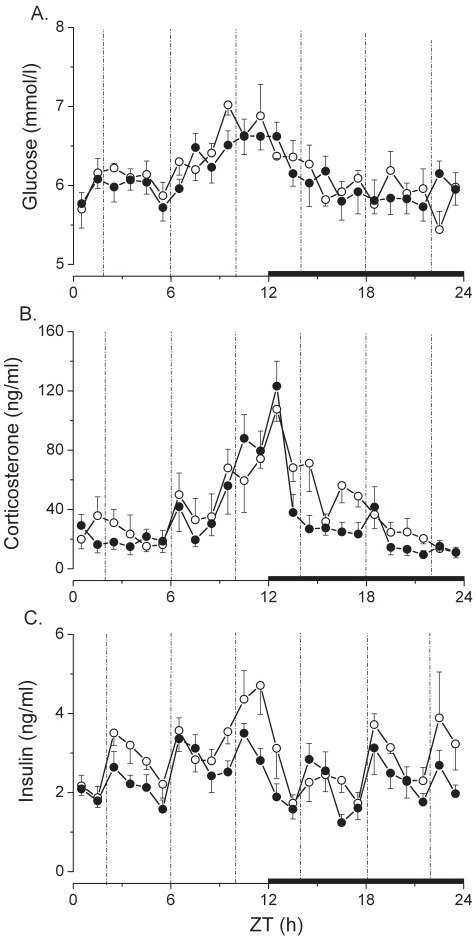

Figure 5 Plasma glucose, plasma insulin and plasma corticosterone concentrations across the light/dark cycle in CD (●; n=7) and sham-operated rats (o; n=5) subjected to a scheduled feeding regimen. The data are represented as the mean ± S.E.M. The black bars indicate the dark period. The vertical dot lines represent the times of food availability (10 min).

variation along the L/D cycle (Figure 5A) ($p<0.001$) with a peak occurring at ZT= 10.61± 0.97 (Goodness of fit (R^2) = 0.35 ± 0.13). No significant effects of *group* and *group*time* were detected, showing that the plasma glucose concentrations in CD did not differ from those of the sham-operated group.

Plasma corticosterone concentrations The 24h profiles of plasma corticosterone concentrations (Figure 5B) measured in the sham-operated rats showed a significant variation along the light/dark cycle ($p<0.001$) as previously described [92, 100] with a peak at ZT12.68 ± 0.71 (R^2= 0.48 ± 0.16). No effect of *Group* was detected by repeated-measure

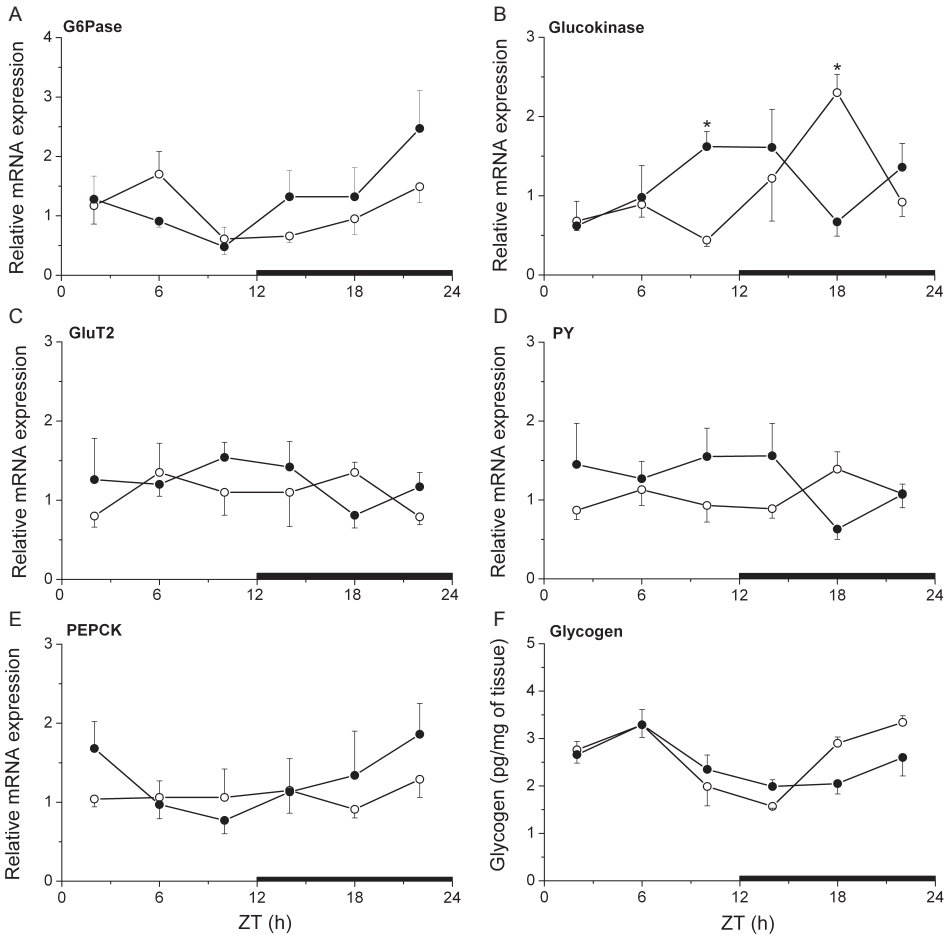

Figure 6 Daily profile in glucose-metabolizing enzyme and hepatic glycogen content in CD rats subjected to a scheduled feeding regimen (●; n = 5 for ZT10/18 and 22, n = 4 for ZT14, n = 3 for ZT2 and n = 6 for ZT6) and under *ad libitum* conditions (○; see figure 4). The data are represented as the mean ± S.E.M. The black bars indicate the dark period. In both groups, some mRNA transcript levels displayed a rhythmic variation across the L/D-cycle (ANOVA, $p<0.05$). *, $p<0.05$ compared to the control group. For statistics see Table S2.

ANOVA, indicating that the complete denervation did not affect the daily pattern in plasma corticosterone concentrations in CD rats. The rhythm showed a peak at the beginning of the dark period (ZT 11.44 ± 0.41) and an R^2 of 0.34 ± 0.11.

An effect of *groupe*time* indicated a slight shift in the daily rise in plasma corticosterone levels in CD groups. Furthermore, the 24h-mean plasma corticosterone concentrations in CD rats were not significantly different from those of sham-operated rats (36.73 ± 4.97 mmol/l vs 40.67 ± 18.19 mmol/l).

Plasma insulin concentrations The basal insulin concentrations in sham-operated and CD animals (Figure 5C) showed a variation over the light/dark cycle ($p < 0.01$), with a clear increase at each meal time. Two way ANOVA did not disclose an effect of *group* and *group*time*, indicating that the denervation does not affect the insulin pattern over the L/D cycle. Moreover, the 24h-mean plasma insulin concentrations in CD rats is similar to those of sham-operated rats (2.35 ± 0.19 mmol/l vs 2.91 ± 0.25 mmol/l).

EFFECT ON METABOLIC ENZYME EXPRESSION/ GLYCOGEN CONTENT

To investigate the influence of food intake without interference of the ANS the 24h-profile of liver metabolic components were measured in CD animals subjected to a scheduled feeding regimen and compared to those obtained in CD animals fed *ad libitum* (Figure 6). The daily pattern in *G6Pase* mRNA expression is maintained in both feeding conditions, but the peak values occur at different times of the day, i.e. at ZT6 and ZT22 for *ad libitum* and regular feeding animals groups respectively (Figure 6). In the same way, we observed a phase-advance in the daily mRNA expression of *GK* in the CD animals with a-rhythmic feeding compared to the *ad libitum* fed animals (*group*time* effect, $p<0.001$). Besides *GLUT2* and *PY* transcript levels, *PEPCK* gene expression did not show a significant effect of *time*, confirming that the ANS is essential for the circadian gene transcription of *PEPCK* (Table S2). Interestingly, the diurnal variation in glycogen content is not affected by denervation and a-rhythmic food intake, indicating that another rhythmic signal might be responsible for the glycogen breakdown/storage rhythm. However, the weaker oscillation observed in the regular feeding group ($p = 0.028$ and $p<0.001$ in 6 meals and *ad libitum* groups, respectively) does indicate that food intake contributes to the daily fluctuation of the hepatic glycogen content.

In summary, a daily rhythm in food intake does not seem an important contributor to the rhythmic expression of metabolic liver enzymes, although it does seem to be able to entrain the *GK*/G6ase rhythms.

Conclusion

Even when direct brain-liver connections are completely absent, the day-night rhythm in plasma glucose levels is still maintained, indicating that the ANS input to the liver is not absolutely essential for the genesis of a daily rhythm in plasma glucose levels. The small changes in the daily profile in plasma glucose levels observed in denervated animals fed *ad libitum* do suggest a contribution of the ANS in the adjustment of the peak at the appropriate time of the day. Combining the complete hepatic denervation with regular feeding, we observed that rhythmic food intake is not essential to drive the daily glucose rhythm either. Together these results indicate the existence of yet other pathway(s) by which the SCN conveys its timing signal to drive the glucose rhythm, for instance via the oscillatory variation of plasma corticosterone levels. How-

ever, in spite of the presence of a clear corticosterone peak, HSx animals subjected to regular feeding lost their rhythmic plasma glucose concentration and hepatic enzyme rhythms. These results indicate that, contrary to the complete absence of ANS input, a dysbalance of the ANS input (provided by the denervation of one branch of the system) may lead to an abnormal glucose metabolism, resulting in a loss of rhythmic plasma glucose levels.

Autonomic regulation of blood glucose rhythm

In contrast to our hypothesis, here we demonstrate that, also without autonomic innervation of the liver, a daily variation in plasma glucose levels can be maintained. Indeed, complete liver denervated animals display a clear 24h-rhythm in plasma glucose concentrations regardless of their daily feeding pattern (i.e., rhythmic or a-rhythmic food intake). However, we did observe a loss in plasma glucose rhythm in HSx or HPx animals, demonstrating that removal of one branch of the ANS input to the liver triggers abnormal glucose metabolism. Interestingly, a sympathovagal imbalance has been proposed and was recently reported as an important mechanism underlying metabolic diseases such as obesity, type II diabetes and metabolic syndrome [233-235]. These studies report that sympathetic over-activity is associated with insulin resistance and increased hepatic glucose production, whereas hyperinsulinemia and fat storage are linked to high parasympathetic tone. Therefore, based on the discovery of the anatomical framework underlying this phenomenon [75, 76, 236], we propose that a dysbalance of the autonomic system, induced by either a hepatic denervation of the sympathetic or parasympathetic branch, leads to an abnormal outflow to the peripheral organs. Interestingly, here we show that indeed an autonomic unbalance induced by HSx affects the liver metabolism; the daily expression in the enzymes *GK*, *G6Pase*, *PEPCK* and the glycogen content are completely disrupted in contrast to complete denervated animals.

In HSx animals, the daily pattern in mRNA expression of *GK* and *G6Pase* does not show an opposite phase, suggesting an abnormal hepatic glucose metabolism, which has also been reported in diabetes and obesity [237-239]. In view of the hypothesis that it is especially the dysbalance of autonomic outflow to the liver that leads to a disturbed liver metabolism, it is logical to observe a sustained 24h-rhythm in plasma glucose levels in completely denervated animals. Our results obtained after complete hepatic denervation are in agreement with studies performed after liver transplantation in which no abnormal liver metabolism has been reported, confirming that the ANS is not essential for hepatic glucose metabolism [240, 241]. All these observations together lead us to propose that it is not the absence, but rather the dysbalance of the ANS that induces an abnormal glucose metabolism in the liver, which in turn results in a loss of the daily plasma glucose rhythm.

SCN uses multiple mechanisms to control liver metabolism in a circadian manner

Whereas HSx induced an abnormal profile in mRNA expression of liver enzymes it did not abolish the oscillatory pattern of the clock gene expressions, the main components of the liver oscillator [217]. Therefore, we propose that the circadian expression of the glucose metabolizing enzymes and the rhythmic glycogen content are underlying the 24h-rhythm in plasma glucose levels and that the clock gene expression in the liver is not sufficient to sustain (or is unrelated to) the rhythmic expression of metabolic enzymes.

The loss of the daily profile in *PEPCK* gene expression observed in CD animals fed *ad libitum* indicates that the ANS participates in the circadian regulation of liver metabolism by generating a rhythmic *PEPCK* gene transcription. The oscillatory pattern of *G6Pase* and *GK* mRNA expression and hepatic glycogen content are in phase with the daily profile of the food intake, but are disturbed by the feeding-schedule, suggesting that these liver enzymes respond to the daily changes in food intake [112]. Consequently, the oscillatory profile of these 3 metabolic parameters (i.e., *G6Pase*, *GK* and glycogen) appears to be sufficient to generate the rhythmicity of plasma glucose concentrations. However, surprisingly, also combining a complete denervation of the liver with a scheduled feeding regimen did not abolish the 24h-rhythm in plasma glucose levels. In agreement with what we proposed for food intake and *G6Pase*/*GK* mRNA levels, CD animals subjected to a 6-meal schedule showed disturbed patterns of *G6Pase*/*GK* mRNA expression. However, their change in expression did not disturb the daily variation in plasma glucose levels, indicating that the rhythm in *G6Pase* and *GK* gene expression is not essential for the glucose rhythm either.

Interestingly, the hepatic glycogen content still exhibits a circadian profile in CD animals subjected to regular feeding, showing that the enzymes involved in the storage/breakdown of glycogen are controlled in a circadian manner, independent of *GK*, *G6Pase* and *PEPCK*. In addition, since glycogen depletion and the daily rise in plasma glucose levels occur at the same time of the day, it is tempting to suggest that the daily variation in hepatic glycogen is the primary cause of the glucose rhythm. The daily rhythm in liver glycogen content is driven by a balance between the activities of glycogen-phosphorylase and glycogen-synthase [242]. So the next question is which rhythmic signal the SCN provides to generate this daily rhythm in hepatic glycogen content. The daily rhythm in plasma corticosterone, which has been shown to be a powerful circadian signal for the liver [189], appears to be a potential factor, since its daily rhythm is not affected by either regular feeding or ANS denervation, and its daily peak coincides with the rise in plasma glucose concentrations. However, the observed absence of a glucose rhythm in the presence of a corticosterone rhythm in HSx or HPx animals seems to contradict this conclusion (Figure S3). Clearly in the present

paper, we have not measured the activity of the liver enzymes and how this activity may compensate for the lack of rhythm in mRNA expression of the same enzymes. In spite of the fact that we are aware of the regulation in enzyme activities [185, 243], the result of CD and single denervation still indicate that the loss of mRNA rhythmicity coincides with a loss of the glucose rhythm, suggesting that enzyme activity will not influence the conclusion of the present study.

In summary, the biological clock uses multiple pathways in order to drive liver metabolism in a circadian manner, independently of local clock genes, autonomic liver innervation and food intake. This shows the extreme capacity of the liver (and SCN) to employ and use other signals to drive its metabolic enzymes in a rhythmic fashion. The most striking observation in this respect is that a dysbalanced autonomic input to the liver is more harmful for a rhythmic glucose metabolism than its complete absence, and is even able to block the strong synchronizing signal of the daily peak in plasma corticosterone.

Acknowledgements

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Supplemental data

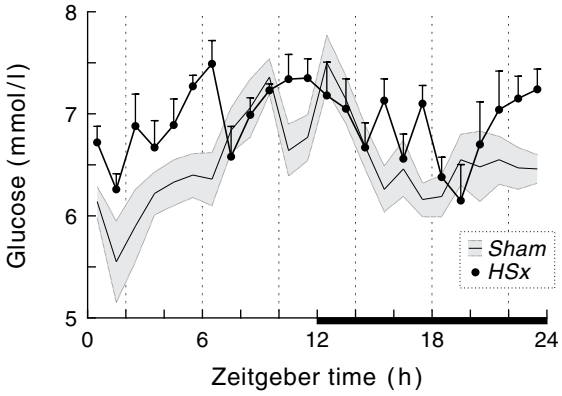

Figure S1 Plasma glucose concentrations across the light/dark cycle under scheduled feeding conditions in HSx (n = 7) and sham-operated rats (n = 7) from our prior study [217]. The gray areas indicate the mean ± S.E.M plasma glucose concentrations in sham-operated animals. The black bar indicates the dark period. The vertical dot lines represent the times of food availability (10 min).

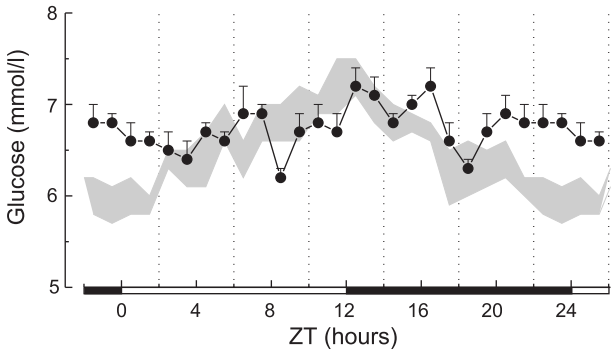

Figure S2 Plasma glucose concentrations across the light/dark cycle under scheduled feeding conditions in HPx and intact rats (extracted from the thesis of La Fleur, 2001). The gray areas indicate the mean ± S.E.M plasma glucose concentrations in sham-operated animals. The black bar indicates the dark period. The vertical dot lines represent the times of food availability (10 min).

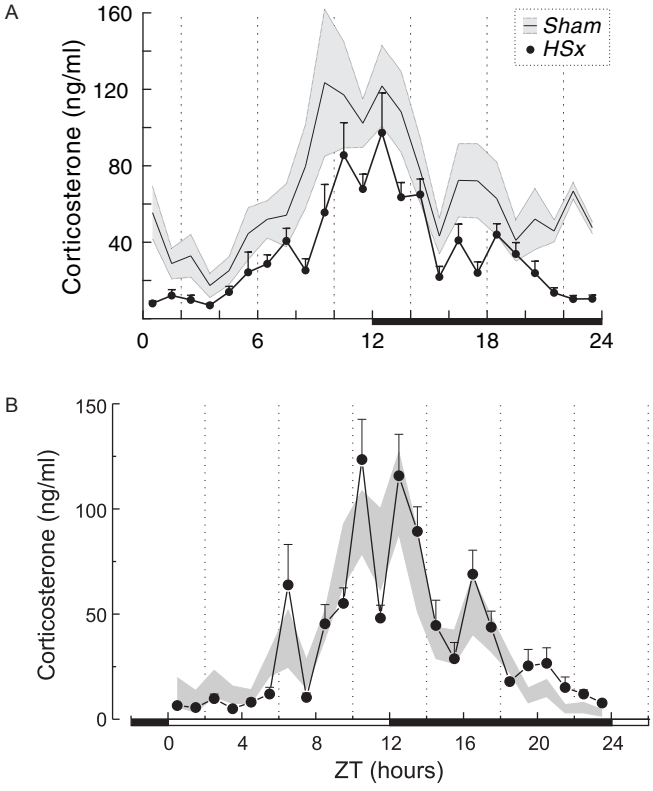

Figure S3 A. Plasma corticosterone concentrations across the light/dark cycle under scheduled feeding conditions in HSx and sham-operated rats from our previous study [217]. B. Plasma corticosterone concentrations across the light/dark cycle under scheduled feeding conditions in HPx and intact rats (extracted from the thesis of La Fleur, 2001). The gray areas indicate the mean ± S.E.M plasma glucose concentrations in sham-operated animals. The black bars indicate the dark period. The vertical dot lines represent the times of food availability (10 min).

Table S1 Statistical analysis of the daily variations of hepatic enzyme mRNA expression and glycogen content in sham/completely denervated rats under *ad libitum*.

			Two-way ANOVA		
		Single	**Time (T)**	**Group (G)**	**Interaction (G x T)**
G6Pase	Sham	= 0.19	= 0.013	= 0.085	= 0.768
	CD	= 0.12			
GK	Sham	= 0.01	≤ 0.001	= 0.022	= 0.577
	CD	≤ 0.001			
GLUT2	Sham	= 0.72	= 0.72	= 0.289	= 0.606
	CD	= 0.46			
PY	Sham	= 0.29	= 0.1	= 0.09	= 0.69
	CD	= 0.32			
PEPCK	Sham	= 0.02	= 0.073	= 0.494	= 0.347
	CD	= 0.9			
Glycogen	Sham	≤ 0.001	≤ 0.001	= 0.004	= 0.002
	CD	≤ 0.001			

One-way ANOVA shows the significant diurnal variations of the enzyme mRNA expressions in both experimental groups. Two-way ANOVA discloses the significance of the difference between sham and completely denervated group.

Table S2 Statistical analysis of the daily variations of hepatic enzyme mRNA expression and glycogen content in completely denervated rats under *ad libitum* and 6-meal schedule.

			Two-way ANOVA		
		Single	**Time (T)**	**Group (G)**	**Interaction (G x T)**
G6Pase	Ad libitum	= 0.12	= 0.02	= 0.36	= 0.21
	6 meals	= 0.038			
GK	Ad libitum	≤ 0.001	= 0.07	= 0.48	≤ 0.001
	6 meals	= 0.115			
GLUT2	Ad libitum	= 0.46	= 0.629	= 0.277	= 0.226
	6 meals	= 0.322			
PY	Ad libitum	= 0.32	= 0.894	= 0.141	= 0.038
	6 meals	= 0.197			
PEPCK	Ad libitum	= 0.9	= 0.31	= 0.232	= 0.542
	6 meals	= 0.226			
Glycogen	Ad libitum	≤ 0.001	≤ 0.001	= 0.34	= 0.11
	6 meals	= 0.028			

One-way ANOVA shows the significant diurnal variations of the enzyme mRNA expressions in both experimental groups. Two-way ANOVA discloses the significance of the difference between *ad libitum* and 6-meal schedule group.

Chapter 4

PVN stimulation affects clock gene and glucose-metabolizing enzyme expression in the liver: Does the SCN use the autonomic nervous system to drive liver metabolism in a circadian manner?

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Submitted

Abstract

Daily plasma glucose concentrations display a peak just before the onset of the nocturnal activity period. Previously we demonstrated that the suprachiasmatic nucleus (SCN) controls this daily fluctuation in plasma glucose concentrations, independent of its influence on feeding activity. Recently, we showed that hyperglycaemia, triggered by an infusion of GABAergic antagonist at the level of the paraventricular nucleus (PVN) of the hypothalamus, is abolished after hepatic sympathectomy (HSx), suggesting that the SCN needs the sympathetic input to the liver to generate the daily rise in plasma glucose concentrations. Besides clock genes, many sugar-metabolizing enzymes in the liver also exhibit a daily rhythm in their gene expression, indicating a circadian transcriptional regulation of metabolic components in the liver. These data suggested that, via its control of the ANS, the SCN could influence the clock gene rhythm in the liver and thereby affect metabolic enzymes in order to generate a daily rhythm in glucose output. Indeed, PVN stimulation by administration of a GABA-antagonist during the light period induced an increase of both the metabolic enzyme and clock gene mRNA expression within the liver, simultaneous with a rise in plasma glucose concentrations. Surprisingly, HSx rats, with only Ringer infusion, showed an increase of some enzymes and clock gene transcripts as well, indicating that the denervation *per se* affects liver metabolism and its clock gene expression. Together, these results indicate the potency of the sympathetic innervation of the liver to participate in the synchronization of the daily rhythms in metabolism.

Introduction

The control of glucose homeostasis is based on a multifaceted mechanism of different tissues, pancreatic hormones, fat-derived hormones, autonomic nervous system and the liver, which all play a major role in regulating glucose metabolism. Besides the tight regulation to keep blood glucose levels within narrow limits, anticipation of increased glucose needs before the onset of the activity period represents another strategy to maintain/control the glucose homeostasis [5, 90, 92, 201]. The biological clock,

located in the suprachiasmatic nucleus (SCN), controls the daily rise in plasma glucose concentrations independent of feeding activity [92]. Nevertheless, it is not clear exactly how the circadian clock controls and generates the 24h-rhythm in blood glucose levels. Retrograde tracing studies using a pseudorabies virus (PRV), demonstrated connections between the circadian clock and the liver via the autonomic nervous system [154], suggesting that the SCN controls the daily glucose metabolism via the liver. In line with this hypothesis, blockade of the inhibitory GABA-ergic SCN input to the PVN during daytime, by infusion of a GABA-antagonist, results in an increase of plasma glucose levels. Furthermore, this hyperglycemia is completely abolished after a sympathetic denervation of the liver, indicating that the SCN might elicit the daily rise of plasma glucose levels (possibly by an increase of hepatic glucose production) by activation of the sympathetic innervation of the liver [73]. The existence of oscillating clock genes in the liver and the presence of a circadian pattern in the expression of key enzymes involved in glucose metabolism (e.g., Phosphoenolpyruvate carboxykinase (*PEPCK*), Glucose-6-Phosphatase (*G6Pase*)) suggest that the SCN uses the liver oscillator in order to adapt the hepatic glucose output according to the time of the day [161, 218, 219, 244]. To determine the importance of clock-gene functionality to generate a rhythmic hepatic glucose-output, we decided to investigate whether SCN-activation of the sympathetic input to the liver affects liver clock gene expression. Resetting the liver oscillator might, in turn, modify liver glucose output by modifying the expression of enzymes involved in hepatic glucose production. Therefore, we reproduced the rise in plasma glucose concentrations by infusion of GABA antagonist at the level of the PVN and we quantified, in the liver of these animals, the mRNA expression of clock genes and enzymes involved in glucose metabolism. Subsequently, we repeated these experiments in HSx animals to investigate the involvement of the autonomic nervous system in these changes.

Materials & Methods

Animals

Adult male Wistar rats (Wu, Harlan, Horst, The Netherlands) were kept in individual cages in a 12 h light/ 12 h dark cycle (L/D; lights on at 7:00, considered as Zeitgeber time (ZT) 0) under constant conditions of temperature (20 ± 2°C) and humidity (60 ± 2%). Food and water were available *ad libitum*, except during the experimental session, when only water was available. All experiments were conducted with the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Surgical procedure

Surgery was performed in rats of 300-350g. Rats were anesthetized using a mixture

of Hypnorm (Janssen, 0.05ml/100g BW, i.m.) and Dormicum (Roche Nederland, 0.04 ml/100g BW, s.c.). During surgery, the abdominal cavity was bathed regularly with saline to prevent drying of the viscera. The wound was closed with atraumic sutures (5-0 Perma-Hand Said Ethicon). After surgery, the animals were placed in an incubator until awakening (temperature 30°C); saline was injected subcutaneously to prevent dehydration. Post-operative care was provided by subcutaneous injection of the painkiller Temgesic (Schering-Plough (Utrecht importer), 0.01ml/100g BW), after the operation and on the following day. A recovery period of at least days 10 days was given to the animals to reach their pre-operative body weight and allow the complete reinstatement of circadian rhythms [245].

HEPATIC SYMPATHECTOMY (HSX)

A laparotomy was performed in the midline. In order to access the bile duct and the portal vein, the liver lobes were gently pushed up and the ligaments around the liver lobes were carefully detached. The bile duct was isolated from the portal vein complex. At the level of the hepatic portal vein, the hepatic artery splits into the hepatic artery proper and the gastro-duodenal artery. At this point, the arteries were separated from the portal vein. The nerve bundles running along the hepatic artery proper were visualized using a myelin-specific dye (Toluidin Blue) and were removed using micro-surgical instruments under an operating microscope (25x magnification). Any connective tissue attachments between the hepatic artery and the portal vein were also broken, eliminating any possible nerve crossings. The sympathetic denervation involves an impairment of both efferent and afferent nerves. However, this procedure does not impair the vagal input to the liver, as shown previously [73, 217].

JUGULAR VEIN CATHETER/INTRACEREBRAL MICRODIALYSIS PROBE

During the surgical procedure (sham or HSx), also an intra-atrial silicone cannula was implanted through the jugular vein according to the methods of Steffens [203], to perform blood sampling. To administrate drugs or Ringer at the level of the PVN, bilateral microdialysis probes were stereotaxically implanted directly lateral to the PVN (coordinates with flat skull: 1.8 mm caudal to Bregma, 2.0 mm lateral to the midline, 7.9 mm below the brain surface). The loop of the U-shaped probe was placed in the rostrocaudal direction along the PVN. At the end, dental cement was used to fix the probe and the atrial outlet to two stainless steel screws inserted in the skull [73, 246]. To familiarize the animals with the experimental procedure, the animals were handled during the period of recovery and sham blood was sampled (i.e., blood was withdrawn and immediately returned) regularly.

Microdialysis probes

Microdialysis probes were constructed according to the method described previously [73, 246]. Thin platinum wire (ø, 0.05 mm) was inserted into a piece of microdialysis tubing

(molecular weight cutoff, 6000 kDa) and bent; each end was then inserted into one of two pieces of 25 gauge hypodermic tubing that had been soldered together. The U-shaped tip of the dialysis probes was 1.5 mm long, 0.7 mm wide and 0.2 mm thick.

Experimental protocol

GENERAL PROCEDURE

At least 48h before the experiment, animals were connected to blood sampling and microdialysis lines, which were attached to a metal collar and kept out of reach to the rats by a counterbalanced beam. The input port of the dialysis probe was connected to a remote syringe via a fluid swivel (375/22; Instech Laboratories, Plymouth Meeting, PA) interconnected with polyethylene tubing. Depending on the experimental group, the syringe contained Ringer's solution with or without drug (Bicuculline (BIC), a $GABA_A$ antagonist; 100μM). The two hours of BIC infusion (or Ringer solution for the control groups) at ZT 4 was preceded by two hours of Ringer's perfusion (with a flow rate of 3μl/min). For more details of the procedure see [73]. Blood samples were taken 5min before and then 5, 30, 60 and 120 min after the onset of the drug infusion.

EFFECT OF THE GABA ANTAGONIST ADMINISTRATION IN THE PVN ON THE EXPRESSION OF LIVER ENZYME MRNA IN INTACT RATS

With this first experiment, we aimed to identify for which enzymes (i.e., key enzymes of the main pathways involved in hepatic glucose production) the mRNA expression was affected by the BIC infusion at the level of the PVN. Therefore two groups of rats were investigated: (1) as experimental group, intact animals were infused with a BIC solution, n = 17, (2) as control group, intact animals were infused with a Ringer's solution, n = 10. Animals were sacrificed at the end of the 2-h of drug infusion.

EFFECT OF GABA ANTAGONIST INFUSION IN THE PVN ON THE EXPRESSION OF LIVER ENZYME MRNA IN HSX RATS

As we had already demonstrated that a sympathetic denervation of the liver abolishes the rise in plasma glucose levels induced by BIC in an earlier study, our aim this time was to identify the metabolic enzymes that are affected specifically by liver denervation, which in turn leads to a loss of the rise in plasma glucose levels. For this purpose two additional groups of animals were investigated: (3) as an experimental group, HSx animals were infused with BIC, n = 16 and (4) as a control group, HSx animals were infused with Ringer's solution, n = 13. Animals were sacrificed 120 minutes after the onset of the 2h drug administration.

High Performance Liquid Chromatography-Electrochemical Measurements

For verification of the completeness of sympathectomy, parts of the liver were processed using High Performance Liquid Chromatography (HPLC) with electrochemical detection for noradrenaline (NA) content. Briefly, tissue samples were weighed and

homogenized in ice-cold 0.1M perchloric acid (2ml/100mg) and centrifuged twice (14000 rpm) for 15 minutes at 4°C. Samples of 20µl supernatant were subsequently transferred to the HPLC for analysis (Waters 600E pump and Waters 717plus autosampler, Waters Chromatography b.v., Netherlands; Decade VT-03 electrochemical detector, Antec Leiden, Netherlands and Shimadzu Class-vp ™ software v.5.03 Shimadzu Duisburg, Germany). The mobile phase consisted of 0.06mol/l Natrium Acetate, 9mmol/l Citric acid, 0.37mmol/l Heptasulfonic acid and 12.5% methanol. The flow rate was kept constant at 0.3ml/min. Separation of NA from other endogenous compounds was achieved with a Supelcosil column (LC-18-DB 25cm x 4,6mm x 5 µm), with a 2 cm guard-column of the same material (Supelco Superguard ™, Supelco USA). Column temperature was kept constant at 28°C. Quantification was achieved by means of electrochemical detection (+0.65V) and NA content was measured against a calibration curve of an external NA standard. The detection limit (ratio signal to noise = 2) was 1 pg of NA injected on column.

Rat liver RNA extraction and cDNA synthesis

Total RNA was extracted from each liver collected by RNeasy mini Kit (Qiagen, Courtaboeuf, France), including DNase step, according to the manufacturer's instructions. For the cDNA synthesis, 2µg of total RNA was reverse-transcribed (125ng of random hexamer/µg of RNA, Hexanucleotide Mix, Roche, Life Technology Nederland) and 10mM of dNTPs) with Superscript II RNase H^- Reverse transcriptase (SSIIRT, In Vitrogen, Life Technology Nederland) according to the manufacturer's instructions. As a control (No reverse-transcriptase (NoRT)), for the detection of unspecific amplification, 2 µg total RNA of each samples followed the same procedure, except that 1µl of the enzyme was replaced by water. Each single stranded cDNA and their respective No RT were then used as a template for the Real-Time PCR.

Real-Time PCR (RT-PCR)

Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). Primer data are given in Table 1. mRNA expression of enzymes involved in glucose metabolism [Glucose 6 phosphatase (*G-6-P*), Glucokinase (*GK*), Glucose transporter 2 (*GLUT2*), Phosphoenolpyruvate Carboxykinase (*PEPCK*)], clock gene [Period 1/2/3 (*Per1/2/3*), *Cryptochrome* 1 (*Cry1*) and *D* site of albumin promoter-binding (*Dbp*; a clock-controlled gene)] and two reference genes [(ubiquitin conjugate enzyme (*Ubc2e*) and TATA box binding protein (*Tbp*)] were analyzed by RT-PCR.

One µl of each cDNA was incubated in a final volume of 20µl RT-PCR reaction containing 1X SYBR-green master mix (Applied Biosystems) and 6 pmol (reverse + forward) of each primer. Control PCRs were performed using PCR reaction mix,

Table 1 Gene nomenclature, GenBank accession code, primer sequences and primer efficiency.

Gene	GenBank	Forward primer	Reverse primer	Efficiency
G6Pase	NM 013098	CCCATCTGGTTCCACATTCAA	GGCGCTGTCCAAAAAGAATC	1.95
GK	NM 012565	TCCTCCTCAATTGGACCAAGG	TGCCACCACATCCATCTCAA	1.98
GLUT2	NM 012879	GAAGGATCAAAGCCATGTTGG	CCTGATACGCTTCTTCCAGCA	1.98
PY	NM 012624	GAGAGTTTTGCAACCTCCCCA	CCTTCACAATTTCCACCTCCG	1.95
PEPCK	NM 198780	TGCCCTCTCCCCTTAAAAAAG	CGCTTCCGAAGGAGATGATCT	2.02
Cry1	NM 198750	AAGTCATCGTGCGCATTTCA	TCATCATGGTCGTCGGACAGA	1.87
Per1	XM 340822	TCTGGTTCGGGATCCACGAA	GAAGAGTCGATGCTGCCAAAG	1.8
Per2	NM 031678	CACCCTGAAAAGAAAGTGCGA	CAACGCCAAGGAGCTCAAGT	1.89
Per3	NM 023978	ATAGAACGGACGCCAGAGTGT	CGCTCCATGCTGTGAAGTTT	2.2
Dbp	NM 012543	CCTTTGAACCTGATCCGGCT	TGCCTTCATGATTGGCTG	2.2
Ubi	U 13176	CTCCAACAGGACCTGCTGAAC	CTGAAGAGAATCCACAAGGAATTGA	1.8
Tbp	XM 217785	TTCGTGCCAGAAATGCTGAA	TGCACACCATTTTCCCAGAAC	1.91

including primers without cDNA (non-template control) or with 1µl of the No RT reaction in the reaction mixture. Quantitative PCR was performed in an Applied Biosystems (Model ABI7300 Prism Sequence Detection System). Cycling conditions were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of: 15 s at 95°C; 1 min at 60°C. The data were acquired and processed automatically by Sequence Detection Software (Applied Biosystems Inc). Primer specificity was confirmed by dissociation (melting) curve analysis and agarose gel electrophoresis. To correct the cDNA load between the different samples, the target gene had to be normalized by a reference gene. Reference genes are selected by their constant expression throughout the different experimental conditions.

Plasma measurements

During the experiment, blood samples were placed in crushed ice in tubes containing 10µl solution of 2.5% EDTA plus 10% benzymidine hydrochloride, before being centrifuged at 4°C. Plasma glucose concentrations were determined in triplicate using a Glucose/GOD-Perid method (Boehringer Mannheim, GmGH, Germany). Plasma corticosterone concentrations were determined in duplicate with a radio-immunoassay kit (ICN Biomedicals, Costa Mesa, CA). From the samples, 10µl was taken and diluted in 4 ml of assay buffer. The lower limit of the assay was 1ng/ml and the coefficient of variation of the immunoassay was less than 4 %.

Statistical analysis

GLUCOSE, CORTICOSTERONE RESPONSES

The responses of plasma glucose and corticosterone during the drug infusion were evaluated by using two physiological parameters: (1) their increase during the 2-h perfusion was compared to their value measured at t = 0, and (2) the area under the curve (AUC) was calculated to estimate the overall changes during the 120 min infusion period. To demonstrate statistically significant hormone responses during PVN administration of drugs (Ringer or BIC) one-way ANOVA with repeated measurements (i.e., 5 blood samples) was used, and to estimate an effect of drug administration (Ringer *vs* BIC) or liver denervation (Sham *vs* HSx) we used multivariate ANOVA (MANOVA) with repeated measures (i.e. sampling, 5 intra-animal levels; groups, 2 inter-animal levels). If a significant difference between two groups was detected, the student's *t*-test (un-paired) was applied to establish at which "time" the significant "group" difference had occurred.

From our previous study [73] we knew that BIC infusion in intact animals with a correct probe placement triggered an increase of plasma glucose and corticosterone with an AUC>100 and >10 000, respectively. Therefore, in the present study only animals with glucose and corticosterone AUC >100 and >10 000 respectively were considered to have a correct probe placement. Since in correct denervated HSx animals the glucose response is abolished [73], here only the corticosterone criterium was applied. The statistical analysis was done on absolute values.

For the data analysis on metabolic enzymes and clock gene expression, animals were selected according to the physiological parameters mentioned above. For the HSx group, animals were additionally selected according to their NA content (i.e. NA content in the liver should be below 15% of the total NA content measured in intact rat liver). To demonstrate a statistically significant difference between the two groups (Ringer *vs* BIC), the P-value of unpaired student's *t*-test was calculated on the basis of the normalized data (SPSS package V12.0).

Results

PVN infusions in intact and HSx rats: Hormonal responses

In 11 out of 17 intact rats, the 2-h BIC infusion at the level of the PVN significantly affected plasma glucose and corticosterone concentrations according to the criteria described above. Glucose and corticosterone responses differed significantly from those found during Ringer's administrations (n = 10), according to ANOVA with repeated measurements (Figure 1; Table 2 and 3).

Concerning HSx rats, 13 out of 16 rats for the BIC group showed a significant increase in plasma corticosterone levels. Seven out of these 13 exhibited a low NA content in the liver and despite a high corticosterone levels they displayed an attenu-

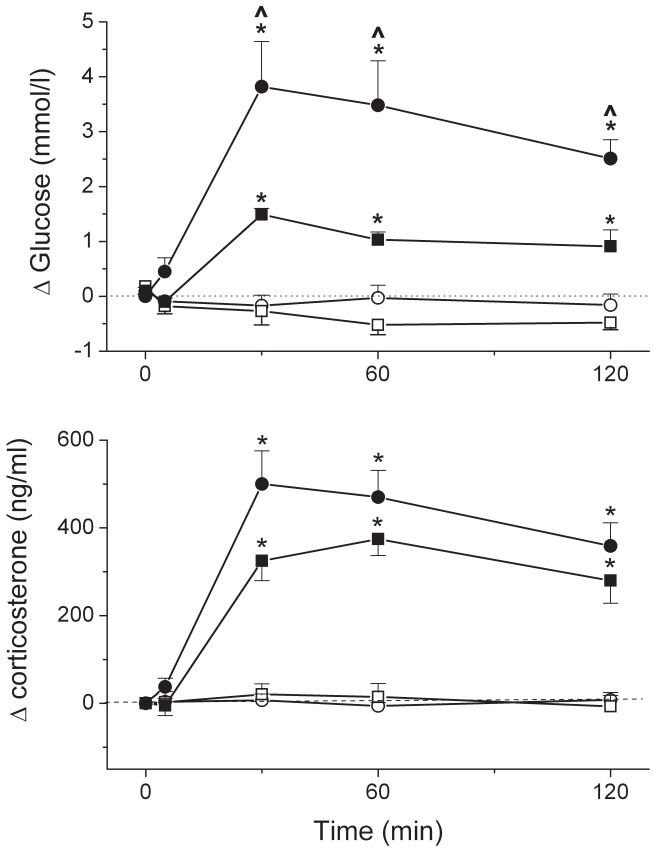

Figure 1 Changes in plasma glucose and corticosterone concentrations during the 2hr administration of Bicuculline and Ringer's solution in the PVN in intact and HSx rats (**circles** and *squares* represent **intact** and *HSx* animals, respectively; open and closed symbols represent Ringer or BIC infusion, respectively). For further details see Tables 2 and 3.

ated rise in plasma glucose (AUC < 100) as previously described [73]. Moreover, 5 out of the 13 rats of the Ringer group were discarded according to the NA criteria and one animal due to a blockage of the jugular vein catheter, thus leaving 7 animals for the Ringer group. A significant effect of *group* and *group*sampling* ($p = 0.018$ and 0.014, respectively) was detected between intact and HSx groups, indicating a different glucose response to BIC infusion depending on the liver innervation, as previously described [73] (Figure 1).

In fact, the HSx rats exhibited only a mild glucose response to GABA-antagonist administration in the PVN as compared to the strong hyperglycaemia observed in

Table 2 Statistical analysis of the plasma glucose response pattern during PVN administration of Ringer or BIC in intact/HSx rats.

			Two-way ANOVA		
		Sampling	**Sampling(S)**	**Group (G)**	**Interaction (G x S)**
Intact	Ringer (n=10)	= 0.71	≤ 0.001	≤ 0.001	≤ 0.001
	BIC (n = 11)	≤ 0.001			
HSx	Ringer (n=7)	= 0.066	≤ 0.001	≤ 0.001	≤ 0.001
	BIC (n= 7)	≤ 0.001			
Ringer	Intact	= 0.71	= 0.052	= 0.22	= 0.426
	HSx	= 0.066			
BIC	Intact	≤ 0.001	≤ 0.001	= 0.018	= 0.014
	HSx	≤ 0.001			

One-way ANOVA with repeated measures (i.e., 5 sampling points) shows the significant variation of the plasma glucose levels during drug administration. MANOVA with repeated measures discloses the significance of the difference between Ringer/BIC group and liver intact/HSx group.

Table 3 Statistical analysis of the plasma corticosterone response pattern during PVN administration of Ringer or BIC in intact/HSx rats.

			Two-way ANOVA		
		Sampling	**Sampling(S)**	**Group (G)**	**Interaction (G x S)**
Intact	Ringer	= 0.66	≤ 0.001	≤ 0.001	≤ 0.001
	BIC	≤ 0.001			
HSx	Ringer	= 0.90	≤ 0.001	≤ 0.001	≤ 0.001
	BIC	≤ 0.001			
Ringer	Intact	= 0.66	= 0.877	= 0.563	= 0.763
	HSx	= 0.90			
BIC	Intact	≤ 0.001	≤ 0.001	= 0.15	= 0.18
	HSx	≤ 0.001			

One-way ANOVA with repeated measures (i.e., 5 sampling points) shows the significant variation of the plasma glucose levels during drug administration. MANOVA discloses the significance of the difference between Ringer/BIC group and sham/HSx group.

intact rats ($p = 0.01$), i.e. AUC 117 ± 14 and 344 ± 62, respectively. In contrast, hepatic denervation did not affect the plasma corticosterone responses, and no significant differences between HSx and Intact groups were detected in the basal concentrations of plasma glucose or corticosterone measured at $t = 0$ (Table 4).

Table 4: Basal concentrations in plasma glucose and corticosterone at the start of PVN infusions.

		Bicuculline	Ringer
Glucose	Intact	6.34 ± 0.17 (n = 11)	6.22 ± 0.22 (n = 10)
	HSx	6.08 ± 0.24 (n = 7)	6.46 ± 0.16 (n = 7)
	p	0.380	0.425
Corticosterone	Intact	62.36 ± 20.73 (n = 11)	39.40 ± 9.69 (n = 10)
	HSx	60.38 ± 22.81 (n =7)	65.7 ± 17.6 (n = 7)
	p	0.560	0.848

Absolute values of plasma glucose and corticosterone concentrations at the start (t = 0) of the infusion experiment. The p-value indicates the results of the unpaired Student's *t*-test.

PVN infusions in intact and HSx rats: effect on glucose enzyme expression

Infusion of a GABA antagonist in the PVN triggered a significant upregulation of all liver enzyme mRNA expressions, except GLUT2 (Figure 2), indicating that the BIC infusion does affect hepatic glucose metabolism. The infusion of BIC at the level of the PVN activates many neurons, which in turn may result in changes in both autonomic activity and hormonal levels such as corticosterone [73]. These autonomic and hormonal changes affect liver metabolism towards a higher glucose output, so it is not surprising to observe an increase in the mRNA levels of glucose-metabolizing enzymes involved in gluconeogenesis correlated with an increase in plasma glucose concentrations. No significant difference was detected between BIC *vs* Ringer groups in HSx animals as far as the enzyme levels were concerned, showing that BIC infusion does no longer affect liver enzyme expression after removal of the sympathetic input. Moreover, in HSx *vs* liver-intact groups with Ringer infusion, we observed that the hepatic denervation *per se* significantly increases the transcript levels of Pepck and PY, the key enzymes involved in the regulation of the gluconeogenic pathway (Table 5). Together these results indicate that the activation of the PVN stimulates the expression of most liver enzymes studied, and this activation is not observed after HSx. Moreover, removal of the sympathetic input to the liver also increases the basal expression of these enzymes.

PVN infusions in intact and HSx rats: effect on clock gene mRNA

BIC infusion at the level of the PVN in intact rats triggered an increase in most of the clock gene transcript levels (Figure 3), except Per3 and the clock-output gene Dbp. The effect of BIC on Per1/2 and Cry1 expression was not observed in HSx animals (Table 6). However, statistical analysis revealed a significant difference between Ringer

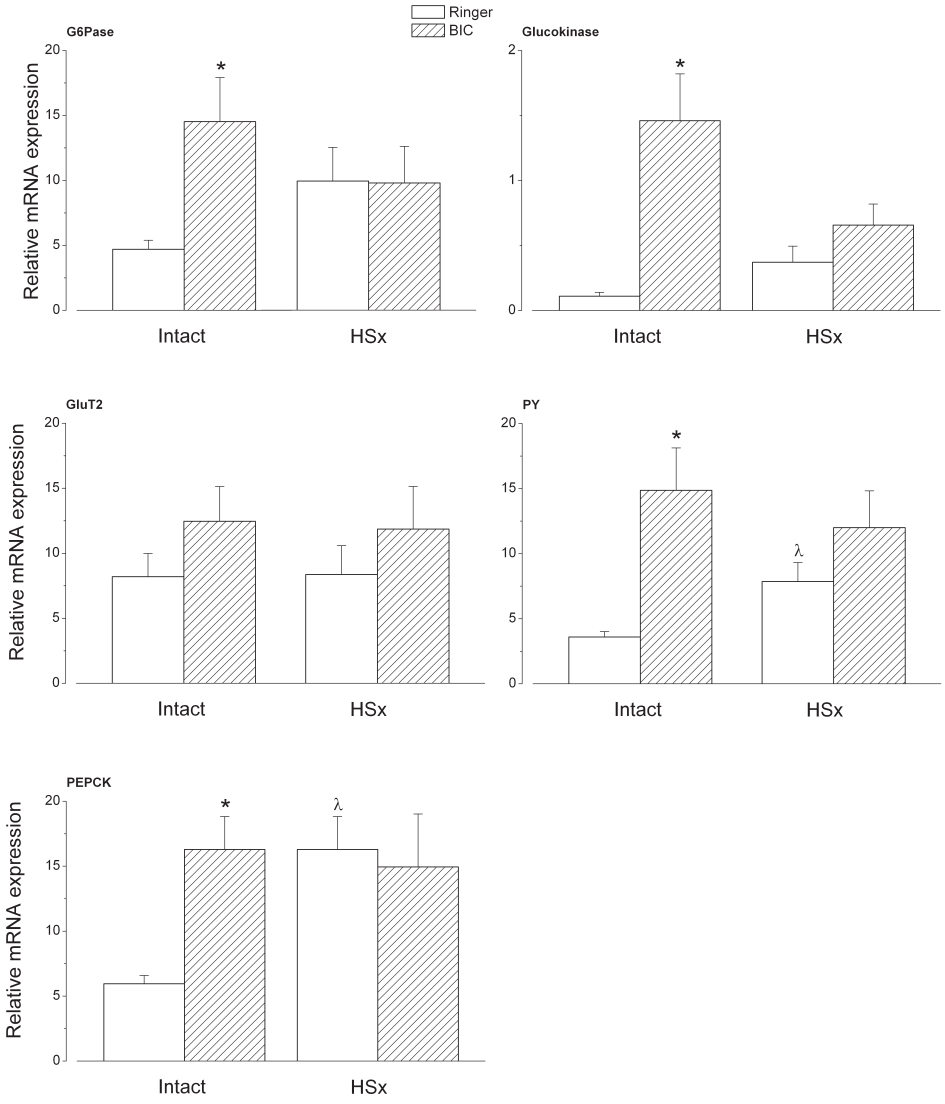

Figure 2 mRNA expression of the liver enzymes after a 2 h infusion of Bicuculline and Ringer's solution in the PVN. * indicates p values < 0.05 for BIC *vs* Ringer; λ indicates p values < 0.05 for Intact *vs* HSx. For further details see Table 5.

infused HSx *vs* liver-intact rats with increased mRNA levels of all clock genes studied, except Dbp. Per3 mRNA levels displayed a significant decrease of mRNA levels in HSx rats upon BIC stimulation. No difference in Dbp gene expression, a clock-controlled gene, was detected in any group, indicating that the clock output was not disturbed.

Table 5 Statistical analysis of liver enzyme mRNA expressions after 2hr of BIC/Ringer administration in the PVN.

		Unpaired Student's *t*-test				
		G6Pase	**Glucokinase**	**GLUT2**	**PY**	**PEPCK**
Intact	Ringer BIC	= 0.01	= 0.002	= 0.211	= 0.004	= 0.001
HSx	Ringer BIC	= 0.97	= 0.18	= 0.39	= 0.22	= 0.78
Ringer	Intact HSx	= 0.24	= 0.33	= 0.29	= 0.023	= 0.006
BIC	Intact HSx	= 0.343	= 0.086	= 0.465	= 0.505	= 0.772

Relative mRNA expression of liver enzymes on completion (t = 120) of the experiments; liver intact group with Ringer (n = 10) or BIC (n = 11) infusion and denervated liver group with Ringer (n = 7) or BIC (n = 7) infusion. The p-value indicates the results of the unpaired Student's *t*-test.

Table 6 Statistical analysis of the liver clock gene mRNA expressions after 2hr of BIC/Ringer administration in the PVN.

		Unpaired Student's *t*-test				
		Per1	**Per2**	**Per3**	**Cry1**	**Dbp**
Intact	Ringer BIC	= 0.011	= 0.005	= 0.172	= 0.021	= 0.893
HSx	Ringer BIC	= 0.38	= 0.14	= 0.01	= 0.211	= 0.315
Ringer	Intact HSx	= 0.015	= 0.019	= 0.001	≤ 0.001	= 0.111
BIC	Intact HSx	= 0.37	= 0.438	= 0.84	= 0.375	= 0.676

Relative mRNA expression of liver clock genes on completion (t = 120) of the experiments; liver intact group with Ringer (n = 10) or BIC (n = 11) infusion and denervated liver group with Ringer (n = 7) or BIC (n = 7) infusion. The p-value indicates the results of the unpaired Student's *t*-test.

Conclusion

The present study provides additional evidence that a GABA-ergic inhibitory mechanism is active on the sympathetic pre-autonomic neurons located in the PVN, in order to control liver metabolism. In view of the demonstrated GABA-inhibitory input from the SCN to the PVN it is possible that this inhibition will vary with the time of the day. Activation of the autonomic neurons by blocking the GABA-ergic input triggers an

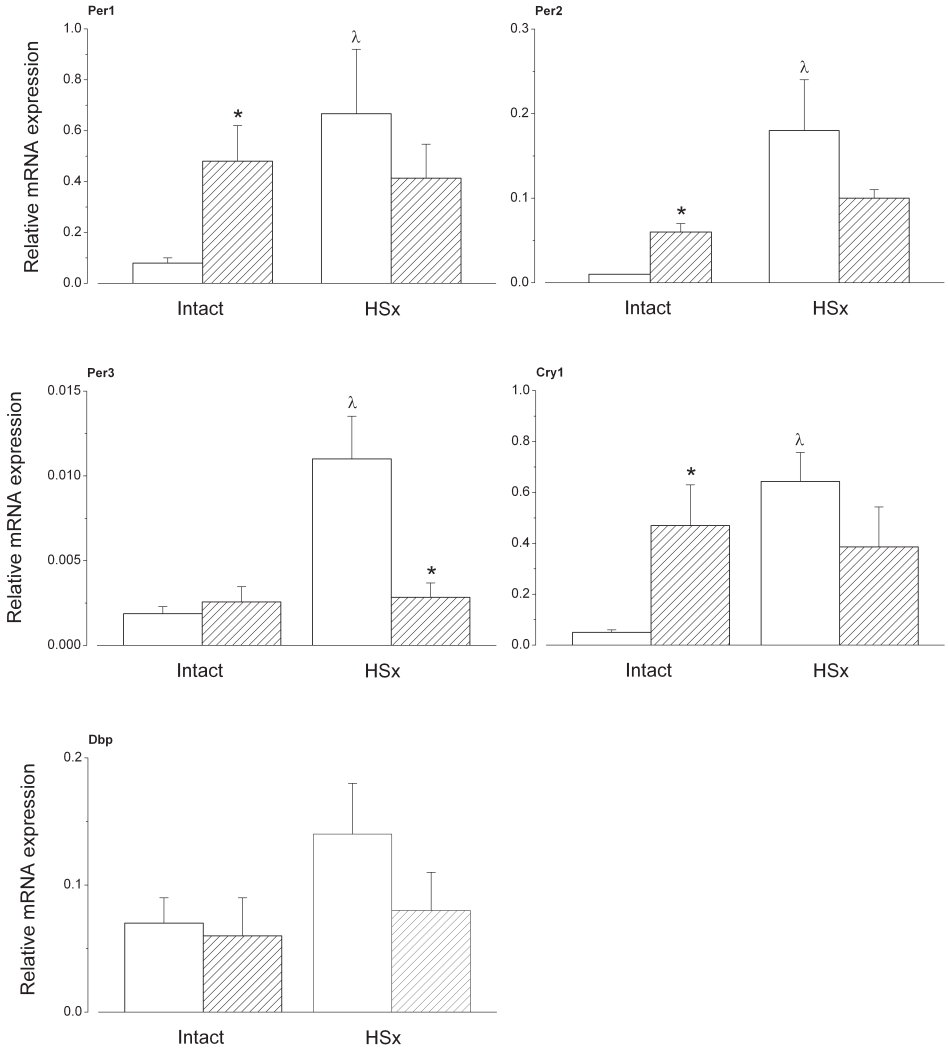

Figure 3 mRNA expression of the liver clock genes after a 2 h infusion of Bicuculline and Ringer's solution in the PVN. * indicates p values < 0.05 for BIC *vs* Ringer; λ indicates p values < 0.05 for Intact *vs* HSx. For further details see Table 6.

upregulation of the gluconeogenic enzyme expression in the liver, which may induce an increase of HGP and then a rise in plasma glucose levels. The BIC-induced upregulation of the liver enzymes and the pronounced hyperglycaemia are abolished after selective sympathetic denervation of the liver, indicating that the increase in plasma glucose concentrations might be due to an increased HGP. Interestingly, we also ob-

served an effect of the hepatic denervation *per se*, i.e., high mRNA levels in metabolic enzymes but no rise in plasma glucose levels or plasma corticosterone levels in animals perfused with Ringer. These data show that a chronic removal of the autonomic input can also affect basal liver glucose metabolism. With regard to a possible time-adjustment of the liver oscillator by the SCN, removal of the SCN-inhibitory input at the level of the PVN does indeed affect liver clock gene mRNA expression, showing that the liver clock genes do respond to an acute change of the SCN-PVN output.

Activation of the autonomic neurones by BIC infusion at the level of the PVN triggers an increase of plasma gluco-regulatory hormone concentrations, known to affect the HGP, i.e., corticosterone, glucagon and catecholamines [73, 108, 247]. Despite the change in these hormonal levels, the BIC-induced rise in plasma glucose levels is strongly diminished after a selective sympathetic denervation of the liver, confirming that the sympathetic innervation of the liver is essential for the BIC-hyperglycemic response, as previously described [73]. The mild rise in plasma glucose levels observed in HSx animals infused with BIC could be explained by the stimulatory effect of corticosterone, glucagon or adrenaline on the hepatic glucose output, which indicates that these hormones participate in, but are not sufficient to generate, the complete glucose response upon BIC infusion in the PVN.

An upregulation of G6Pase/GK and PY enzymes involved in glucose release/uptake and the glycolytic pathways, respectively [185], is observed in liver-intact animals after 2-h BIC infusion in the PVN, indicating that removal of the GABA inhibitory input activates liver metabolism by means of an increase of glucose cycling and glycolysis. The high levels of PEPCK mRNA, the key enzyme of gluconeogenesis, suggest that the hyperglycemic effect of BIC can be attributed to an increased HGP, as previously proposed [202]. It is surprising to observe a stimulatory effect on both glycolysis and gluconeogenesis since their regulation is proposed to be reciprocal [151, 248]. However, in our experiment the analysis of gene expression is performed after 2-h of BIC-induced hyperglycaemia, a "long period of high plasma glucose levels" that might trigger the activation of PY transcription [249]. Moreover, hepatic autoregulatory processes might also take place during this prolonged period of hyperglycemia in order to decrease the plasma glucose levels [250]. In addition to the activation of the autonomic branches connected to the liver, the activation of the autonomic neurones at the level of the PVN induces, the release of hormones which are also known to activate gluconeogenic pathways by inducing gene expression or enzyme activity [108, 153, 251]. Therefore, a selective denervation of the sympathetic input to the liver was performed to identify the set of enzymes which responds specifically to sympathetic nerve stimulation. Interestingly, the hepatic denervation *per se* induces an upregulation of most enzymes implicated in the gluconeogenic pathways, i.e. PECK involved in the regulation of the gluconeogenesis and PY involved in the glycolysis pathway. GK and G6Pase, in-

volved in the uptake/release of glucose and in the replenishment of glycogen [185, 252], also showed increased mRNA levels, but this did not reach significance. The increase of the enzyme mRNA levels observed in HSx control animals (i.e. Ringer infusion in the PVN) is not correlated with an increase in plasma glucose levels, which suggests that the sympathetic input to the liver is also involved in the control of basal hepatic glucose output. However, additional experiments, using a hyperinsulinemic euglycemic clamp, have to be performed in order to be able to confirm or reject this hypothesis. No significant changes of gene expression were observed in the HSx groups after 2h of BIC administration in the PVN, reinforcing the idea that the sympathetic input to the liver is necessary to generate a rise in plasma glucose levels by means of hepatic glucose release. Surprisingly, removal of the sympathetic input to the liver also caused an upregulation in enzyme expression, just like the BIC-induced activation of the sympathetic input, suggesting that the chronic dysbalance of the ANS provides a strong stimulatory signal to the liver comparable to the BIC infusion. A study in which a parasympathetic denervation of the liver was reported to cause a disturbance in liver glucose metabolism as well as glycogen storage [225] also indicates that removing one branch of the autonomic input to the liver may generate an abnormal hepatic glucose metabolism. In a previous study we demonstrated that sympathetic denervation *per se* disturbs the daily rhythm of the metabolic enzymes in the liver, which in turn resulted in a loss of the plasma glucose rhythm (chapter 2). Consequently, this study confirms our hypothesis that a balanced autonomic input to the liver is required to sustain the rhythmicity in hepatic enzyme expression. In the present study, we intentionally measured the expression of the liver enzymes in order to identify the set of metabolic enzymes involved in the daily rhythmicity of HGP. We are, of course, aware of the complex regulation of the liver enzyme activity (i.e., at the pre and posttranscriptional levels) [231, 253-255], and in future experiments we therefore aim to investigate further the activity levels of the enzymes in order to be able to identify more precisely the disturbance of the glucose metabolism that occurs after the sympathetic denervation.

As it was previously reported that sympathetic stimulation caused an increased of Per1 mRNA levels [180], the other question we raised in this study was whether the SCN can use the sympathetic input to reset the liver oscillator in order to control its glucose output according to the time of the day. The removal of the SCN-inhibitory input to the PVN does induce an increase of Per1/2 and Cry1 expression in the liver-intact animals. Since the liver oscillator has been shown to respond to multiple signals [178, 179], the upregulation of Per2 and Cry1might be induced by the BIC-induced hormonal changes or the activation of both autonomic inputs to the liver. Remarkably, as observed for liver enzymes, hepatic denervation alone induces an upregulation of clock genes, indicating that hepatic denervation provides a signal which is perceived by the liver oscillator. This observation is quite surprising, since our previous work

did not yield any major effects of the denervation on the rhythmic profile of the liver clock genes [217]. However, a closer look at the expression level of the clock genes at ZT6 of both intact-liver and HSx animals, from our previous study shows an increasing effect of the hepatic denervation *per se* as well. Taking all this together, it appears that the hepatic denervation *per se* does affect the basal expression of the clock gene without affecting their 24h-rhythmic profiles over the L/D cycle. Interestingly, in HSx animals, BIC infusion induces a significant decrease of Per3 mRNA levels (a similar trend towards a decrease is also observed in other clock gene expressions), suggesting an inhibitory effect of the parasympathetic branch on clock gene expression. At present we are not aware of any other published data showing a possible connection between the parasympathetic branch of the ANS and clock gene expression. On the other hand, it has been reported that light (via the SCN) does have opposing effects on the two branches of the ANS [256].

Recently, Ishida and collaborators demonstrated a role for the sympathetic innervation of the adrenal in light-induced corticosterone secretion and also observed an effect on clock gene expression, suggesting a role of the adrenal oscillator [82]. The correlative results of this study are still insufficient to conclude that the peripheral clock plays a part in rhythmic organ metabolism, and further studies are still needed to elucidate this. However, a light stimulus, in contrast to BIC, might provide us with a better tool to further investigate our question how the ANS is able to affect clock gene and enzyme expression in the liver. On the other hand, according to Schibler & Brown [184], light only has an indirect effect on the liver, via corticosterone secretion by the adrenal.

In summary, the removal of the GABAergic-input at the level of the PVN induced an increase of gluconeogenic enzymes expression that is probably responsible for the rise in plasma glucose concentrations. Selective sympathetic denervation of the liver induces an abnormal hepatic glucose metabolism, which might explain the absence of BIC-induced hyperglycaemia in HSx animals. Therefore, the integrity of the ANS seems to be required to generate a rhythmic hepatic glucose output and is thereby essential for the genesis of the 24h-rhythm in plasma glucose concentrations. On the other hand, with our studies we have not found an essential role of the liver oscillator in the genesis of a rhythmic hepatic glucose output.

Acknowledgements

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Chapter 5

Light affects the functionality of organs via the autonomic nervous system

C. Cailotto, J. Lei, J. van der Vliet, C. van Heijningen, C. Van Eden, A. Kalsbeek, P. Pévet and R.M. Buijs

In preparation

Abstract

The suprachiasmatic nucleus (SCN) regulates the daily rhythms in physiology and behavior via hormonal and autonomic pathways. Light, conveyed via the retinohypothalamic tract, is the main synchronizer of SCN activity. Early studies demonstrated that light can affect the output of the SCN, and hence the hormonal output or the functionality of peripheral organs. The same light stimulus also induces changes in clock gene expression in the adrenal, suggesting that the peripheral clocks play a role in the organ-specific output. In the present study, we show that a light pulse not only affects clock gene expression but also the expression of functional genes in different organs. Furthermore, changes in the expression of functional genes, observed after light exposure, are correlated with a change in the physiological organ-output for some organs, for instance the adrenal. However, we did not find a correlation between changes in the expression of clock genes and functional genes in response to light, suggesting that peripheral clocks do not participate in the physiological response of an organ to light exposure. Because light induces an immediate change in the release of hormones, such as melatonin and corticosterone, these two hormones were proposed to be necessary to spread the light message to the rest of the body. Interestingly, in our study we demonstrate that the response of the liver to light exposure is mediated by the autonomic nervous system, independent of hormonal changes, indicating additional pathways by which the light is able to influence peripheral organs via the SCN.

Introduction

During evolution, time keeping mechanisms such as the molecular biological clock have evolved in all cells of the body. However, in mammals the autonomous biological clock is located in the brain, in the hypothalamic suprachiasmatic nucleus (SCN) [257, 258]. SCN neurons display spontaneous circadian rhythms of electrical activity, *in vivo* as well as *in vitro* [12, 259, 260]. The SCN transmits its daily signal to all tissues of the body via hormones and the autonomic nervous system [52, 92, 261-263] and in this way prepares all body tissues for either activity or sleep.

The neuronal activity of the SCN in night-active rodents coincides with the loco-

motor inactive (light) phase, while SCN neuronal inactivity coincides with locomotor activity. Until now light has been considered the most important factor to influence the neuronal activity of SCN neurons, via the ventral, light input-receiving, part of the SCN. This light input not only inhibits melatonin secretion, but also affects corticosterone secretion, and inhibits locomotor activity and heart rate [51, 52, 264]. The light-induced inhibition of locomotor activity can only be observed in SCN-intact animals, suggesting that the (light-induced) electrical activity of SCN neurons is directly responsible for the inhibition of locomotor activity, also during the subjective light phase. Since the SCN affects both the pineal and the adrenal via sympathetic innervation, and the result is not only a change in hormone secretion, but also in clock gene expression [82, 159], it has been suggested that these two hormones transmit the SCN signal to peripheral tissues in order to control organ function [184, 265-267]. Especially the influence of the SCN on peripheral clock genes is thought to be transmitted by its influence on corticosterone secretion [82, 184]. However, autonomic connections between the SCN and almost every organ of the body have been demonstrated [51, 154, 155, 261-263, 268], and this neuronal route therefore provides an alternative possibility for the SCN to transmit, not only its circadian message, but also light information to the peripheral oscillators, thereby adjusting the organ-output according to the light changes in the environment.

In the present study, we aimed at examining this hypothesis by investigating two main questions: 1) Are the light-induced changes in clock gene expression implicated in the functional changes of an organ? and 2) Is the autonomic nervous system (ANS) necessary for the light-induced changes in clock gene expression and functional output of the peripheral organs? In order to explore whether the peripheral clock genes are involved in the light-response of the peripheral tissues, clock gene expression and functional genes of different organs (i.e., adrenal, liver, pineal, heart and muscle tissue) were analyzed after light exposure at CT14 and CT20. As a physiological response to light, the plasma corticosterone levels were also measured. To examine whether light information is conveyed via the autonomic nervous system, we focused on the expression of clock genes and functional genes in the liver of intact and liver-denervated animals before and after a light pulse.

Materials and Methods

Animals

Male Wistar rats (Harlan Nederland, Horst, The Netherlands) were housed at a room temperature of 21 ± 1 °C with a 12-h light/12-h dark (L/D) cycle (lights on at 07:00 AM, Zeitgeber Time (ZT) 0). Room lights had an intensity of ≈ 200 lux, while under dark conditions the dim red light was <1 lux. Animals were housed four to six per cage and transferred to individual cages (25×25×35cm) after surgery or 1 week prior to the light exposure experiment. Food and water were available *ad libitum*. All of the following

experiments were conducted under the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

To investigate the effect of light exposure via the SCN on physiological output (e.g., corticosterone secretion), on peripheral clock genes and on tissue-specific genes in animals with or without ANS liver innervation, the animals were subjected to light exposure at subjective night. The rats were transferred to constant dark the day before the experiment. In all experiments, the control group followed the same surgical procedure, but received no light exposure.

Surgical procedure

Surgery was performed in rats of 300-350g. During all surgical procedures animals were anesthetized using a mixture of Hypnorm (Duphar, The Netherlands; 0.05ml/100gm body weight, i.m.) and Dormicum (Roche, The Netherlands; 0.04ml/100gm body weight, s.c.). The wound was closed with traumatic sutures (5-0 Perma-Hand Said Ethicon), and postoperative care was provided with a subcutaneous injection of 0.01ml/100g BW of pain killer (Temgesic; Schering-Plough (Utrecht importer). The animals were allowed a recovery period of at least days 10 days so that they could reach their pre-operative body weight and regain their circadian rhythms.

COMPLETE DENERVATION OF LIVER

By using microsurgical techniques and a myelin-specific dye (Toluidin Blue), a complete hepatic denervation (i.e., sympathectomy and parasympathectomy) was performed, after a laparotomy had been performed in the midline. Hepatic sympathectomy (HSx) was performed as previously described [73, 217]. Briefly: the ligaments around the liver lobes were severed to free the bile duct and the portal vein complex. The nerve bundles were identified at the level of the hepatic artery proper and removed using microsurgical instruments. Hepatic parasympathectomy (HPx) was performed as previously described [73, 221]. Briefly, the hepatic branch was revealed where it separated from the left vagal trunk, subsequently the fascia containing the branch was stretched and then sectioned with a pair of scissors.

JUGULAR VEIN CATHETER

A jugular vein catheter was implanted to investigate the hormonal response of the adrenal cortex to light exposure (i.e., corticosterone secretion). During the surgical procedure, an intra-atrial silicone cannula was implanted through the jugular vein according to the method of Steffens [203] to allow stress-free remote blood sampling.

SCN-LESIONS

Bilateral lesions of the SCN were performed as described previously [61, 139] when the animals weighed 175-200g. A total of 40 animals were operated upon to ensure a sufficient number of effectively lesioned animals. After a recovery period of 2 weeks the effectiveness of the SCN lesions was checked by measuring the daily water intake

for a 3-week period. Intact animals normally consume only 0-5% of their total daily water intake during the middle eight hours of the light period, i.e. between ZT2-ZT10. SCN lesions were considered successful when an animal drank >30% of its daily water intake during this 8-h period.

Experimental Set up

EXPERIMENT 1: EFFECT OF LIGHT EXPOSURE ON PERIPHERAL PHYSIOLOGY (CORTICOSTERONE SECRETION)

In the first experiment, liver-intact animals were exposed to light at early subjective night (CT14) for 30 min. Hourly blood samples were taken from CT9 until CT17. Additional blood samples were taken 15, 30, and 45 min after the start of the light exposure at CT14. One week later the experiment was repeated in the same group of animals, but this time no light exposure was performed at CT14.

A second group of animals was exposed to 30 min of light at different times of the L/D-cycle, i.e. CT2, CT10, CT14, or CT20. Animals were in constant darkness for at least 1 complete subjective dark period preceding the light exposure. During each light exposure only 2 blood samples were taken, i.e. the first one just before the start of the 30 min light exposure and the second one at the end. The different light exposure experiments were separated by at least one week. In addition to the intact animals, this experiment also included a group of SCN-lesioned (SCN-X) animals. The final histological analysis revealed 12 animals with complete lesions of the SCN. The SCN-lesioned animals only participated in the CT14 light exposure.

EXPERIMENT 2: EFFECT OF LIGHT EXPOSURE ON GENE EXPRESSION IN PERIPHERAL ORGANS

In order to investigate the effect of light on the various organs, intact animals were exposed to a 60-min light pulse at either CT14 or CT20. A control group of animals was not exposed to light.

To investigate whether the autonomic nervous system participates in the light-induced liver response, animals with a complete liver denervation (CD) were exposed to the 60-min light pulse at CT14. Control animals, either liver-denervated or sham-operated, received no light.

Euthanasia

Animals were sacrificed immediately at the end of the light exposure (at CT15 or CT21). Pineal, adrenal, liver, heart and skeletal muscle (triceps brachii) were removed and frozen directly into liquid nitrogen and transferred to -80°C until analysis.

High Performance Liquid Chromatography-Electrochemical Measurements

From our previous studies we knew that, in contrast to the parasympathetic dener-

vation, it was difficult to get a 100% success rate for the sympathetic denervation [217]. Therefore, we checked the completeness of the sympathectomy by measuring the NA content in the liver. After the experimental procedures pieces of the liver were processed using High Performance Liquid Chromatography (HPLC) with electrochemical detection for noradrenaline (NA) content. Tissue samples were homogenized in ice-cold 0.1M perchloric acid (2ml/100mg) and centrifuged twice (14000 rpm) for 15 minutes at 4°C. Samples of 20µl supernatant were subsequently transferred to the HPLC for analysis (Waters 600E pump and Waters 717plus autosampler, Waters Chromatography b.v., Netherlands; Decade VT-03 electrochemical detector, Antec Leiden, Netherlands and Shimadzu Class-vp ™ software v.5.03 Shimadzu Duisburg, Germany). The mobile phase consisted of 0.06mol/l Natrium Acetate, 9mmol/l Citric acid, 0.37mmol/l Heptasulfonic acid and 12.5% methanol. The flow rate was kept constant at 0.3ml/min. Separation of NA from other endogenous compounds was achieved with a Supelcosil column (LC-18-DB 25cm x 4,6mm x 5 µm), with a 2 cm guard-column of the same material (Supelco Superguard ™, Supelco USA). Column temperature was kept constant at 28°C. Quantification was achieved by means of electrochemical detection (+0.65V) and NA content was measured against a calibration curve of an external NA standard. The detection limit (ratio signal to noise = 2) was 1 pg of NA injected in the column. Sympathetic denervation was considered to be complete when the NA content dropped below 10% of that of intact-liver animals [222].

RNA extraction and cDNA synthesis

Total RNA was extracted and purified by RNeasy mini kit (Qiagen, Courtaboeuf, France) including a DNase step, according to the manufacturer's recommendations. The quality of RNA was examined by Agilent 2100 Bioanalyzer equipped with Nano chips (Agilent Technologies, Palo Alto, CA, USA) and concentrations were determined by Nanodrop spectrophotometer (NanoDrop Technologies, USA). 2µg of total RNA (liver, adrenal and heart) or 0.5µg of total RNA (pineal) was reverse-transcribed in a final reaction volume of 20µl. First, total RNA and random hexamer (125ng/µg of RNA, Hexanucleotide Mix, Roche, Life Technology Nederland) were incubated for 10 min at 70°C. Then, 5X first strand buffer, 0.1M DTT and dNTPs (10mM) were added and the samples were preincubated for 2 min at 42°C. Finally, 1µl of Superscript RNase H^- Reverse transcriptase (SSIIRT, In Vitrogen, Life Technology Nederland) was added and the reaction was incubated for one hour at 42°C. For the control sample (no RT), the 1ul of enzyme was replaced by 1ul of water.

Real-time PCR (RT-PCR)

Clock genes, which are the main oscillator components (Period 1/2/3 (*Per1/2/3*), Cryptochrome 1/2 (*Cry1/2*) and D-albumin-binding protein (*Dbp*)), were analyzed by

Table 1 Primer sequences for RT-PCR

Gene	GenBank	Sequence
Histone 1	X70685	5' GAACGCCGACTCCCAGATC3' 5' CCCCTTTGGTTTGCTTGAGA3'
HPRT	AF009656	5'ATGGGAGGCCATCACATTGT3' 5'ATGTAATCCAGCAGGTCAGCAA3'
GAPDH	NM_017008	5'TGCCAAGTATGATGACATCAAGAAG3' 5'AGCCCAGGATGCCCTTTAGT3'
Ubi	NM_025356	5'CTCCAACAGGACCTGCTGAAC3' 5'CTGAAGAGAATCCACAAGGAATTGA3'
Tbp	XM 217785	5' TTCGTGCCAGAAATGCTGAA3' 5'TGCACACCATTTTCCCAGAAC3'
EF1a	NM_012660	5'AGATGGACTCCACGGAACCA3' 5'GTAGGCGCTGACCTCCTTGAC3'
Per1	AB092976	5'TCTGGTTCGGGATCCCACGAA 3' 5'GAAGAGTCGATGCTGCCAAAG3'
Per2	NM_031678	5'CACCCTGAAAAGAAAGTGCGA3' 5'CCACGCCAAGGAGCTCAAGT3'
Per3	NM_023978	5'ATAGAACGGACGCCAGAGTGT3' 5'CGCTCCATGCTGTGAAGTTT3'
Cry1	NM_007771	5'AAGTCATCGTGCGCATTTCA3' 5'TCATCATGGTCGTCGGACAGA3'
Cry2	NM_133405	5'TGGATAAGCACTTGGAACGGAA3' 5'TGTACAAGTCCCACAGGCGGTA3'
Dbp	NM_012543	5'CCTTTGAACCTGATCCGGCT3' 5'TGCCTTCTTCATGATTGGCTG3'
NAT	NM_012818	5'TGAGCGCGAAGCCTTTATCTC3' 5'TGATGAAGGCCACAAGACACC3'
G6Pase	NM_013098	5'CCCATCTGGTTCCACATTCAA3' 5'GGCGCTGTCCAAAAAGAATC3'
GLUT2	NM_012879	5'GAAGGATCAAAGCCATGTTGG3' 5'CCTGATACGCTTCTTCCAGCA3'
GK	NM_012565	5'TCCTCCTCAATTGGACCAAGG3' 5'TGCCACCACATCCATCTCAA3'
PY	NM_012624	5'GAGAGTTTTGCAACCTCCCCA3' 5'CCTTCACAATTTCCACCTCCG3'
PEPCK	NM_198780	5'TGCCCTCTCCCCTTAAAAAAG3' 5'CGCTTCCGAAGGAGATGATCT3'
MC2-R	AF547168	5'CCTTCTGCCCAAATAACCCTT3' 5'CATGCCATTGACCTGGAAGA3'

RT-PCR. In addition, some tissue-specific gene-relating physiology (arylalkylamine-N-acetyltransferase (*NAT*) in the pineal, melanocortin receptor-2(*MC2-R*) in the adrenal and glucose-metabolizing enzymes in the liver [Glucose 6 phosphates (*G-6-P*), Glucokinase (*GK*), Glucose transporter 2 (*GLUT2*), Phosphoenolpyruvate Carboxykinase (*PEPCK*)]) was also analyzed. RT-PCR was carried out on an Applied Biosystems (Model ABI7300 Prism Sequence Detection System). 1 µl of each cDNA was incubated in a final volume of 20µl RT-PCR reaction containing 10µl of 2 X SYBR-Green master mix, 1.5 µl of each primer at 3pmol µl-1 and 6µl of RNase-free water. Quantitative PCR was performed under the following general PCR conditions: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C; 1 min at 60°C. The data were acquired and processed automatically by Sequence Detection Software (Applied Biosystems Inc). Gene-specific primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA, USA). The Primer data are given in Table 1. The selection of the two reference genes for each kind of organ or tissue used were confirmed by the GeNorm v3.3 program (http://allserv.ugent.be/~jvdesomp/genorm/).

Histone1 (*H1*) and hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) were chosen as reference genes for pineal; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and ubiquitin (*Ubi*) for adrenal, *HPRT* and *Ubi* for heart, TATA box binding protein (*Tbp*) and *Ubi* for liver, and *H1* and elongation factor alpha (*EFa*) for muscle. Primer specificity was confirmed by dissociation (melting) curve analysis and 5% polyacrylamide gel electrophoresis with 25bp ladder.

Plasma corticosterone measurements

Plasma corticosterone concentrations were determined in duplicate with a radio-immuno-assay kit (ICN Biomedicals, Costa Mesa, CA). From the samples, 10µl was taken and diluted in 4 ml of assay buffer. The lower limit of the assay was 1 ng/ml and the coefficient of variation of the immunoassay was less than 4 %.

Statistical analysis

All data are presented as mean ± SEM. Multivariate analysis of variance (MANOVA) with repeated measurement design was used to determine the effect of light on plasma corticosterone concentration. When MANOVA detected a significant effect, this was followed by a student's-Newman-Keuls (*post hoc)* procedure to assess the specific time of the difference. RT-PCR data were analyzed by a mixed one-way ANOVA (Group (2 levels), i.e., CT15 *versus* CT21, or Intact *versus* Denervated; Treatment (2 levels), i.e., light *versus* no-light). If significant effects were detected, it was followed by a post-hoc LSD test. The differences were considered significant when p values were lower than 0.05.

Results

Effects of light exposure on organ output (plasma corticosterone concentration)

When considering the plasma corticosterone curves from CT9 – CT17 (Figure 1A), either with or without light-exposure, no significant differences were observed (n = 9; effect of CT-times, p<0.001; Light, p = 0.958, Interaction, p = 0.261). However, when the 3 additional time points during the light exposure were included in this analysis (n = 9 + 3) the effect of light was clearly present in the interaction effect (CT-times, p<0.001; Light, p = 0.232; Interaction, p = 0.002). These results indicate that light exposure had an immediate, short-lasting effect on the release of corticosterone. The post-hoc analysis revealed that 15 and 30 min after the start of the light exposure plasma corticosterone levels were significantly higher in the light-exposed animals.

The second experiment showed that the effect of light on corticosterone release was both time- and SCN-dependent (Figure 1B). First, ANOVA showed that the basal (t = 0) corticosterone levels in the SCN-intact animals showed the expected diurnal variation (p<0.001), i.e. with the levels at CT10 being significantly higher than those at the other 3 time points, and those at CT14 significantly higher than those at CT2 (data not shown). Basal plasma corticosterone levels in SCN-lesioned animals were intermediate and differed significantly from those in the intact animals at CT2 and CT20. ANOVA also showed a clear time-dependence of the corticosterone response to light exposure, except in the SCN-X animals (Figure 1B). Irrespective of whether SCN-X animals were included or not, the response of intact animals at CT14 differed from all other responses (both p<0.001).

Effects of light exposure on the gene expression in different organs

The purpose of this experiment was to characterize the time dependency of the light-induced changes in the expression of peripheral clocks in different organs. Four groups of rats were either exposed to light at CT14 or CT20 or kept in darkness. Animals were sacrificed at CT15 (n = 8 for controls and n = 9 for light exposed) and CT21 (n = 6 for both groups). The control group at CT15 consisted of intact animals (n = 4) and animals that had been sham-operated (n = 4), since there were no significant differences between intact and sham-operated animals (p > 0.05). In all organs studied, the basal expression of most of the clock genes showed a clear time-dependency (Table 2 and Figure 2 A, B). In fact, only 4 out of the 30 combinations did not show a significant effect of circadian time. The circadian changes observed in our study confirmed those of previous studies [159, 161, 168, 170, 197, 207, 217].

We observed a change in the expression of the functional genes in the liver and the pineal at CT13 and CT15 (except for PEPCK, which displays a low expression during the dark period; Table 2 and Figure 2 C), similar to [166, 219, 244]. Interestingly, the

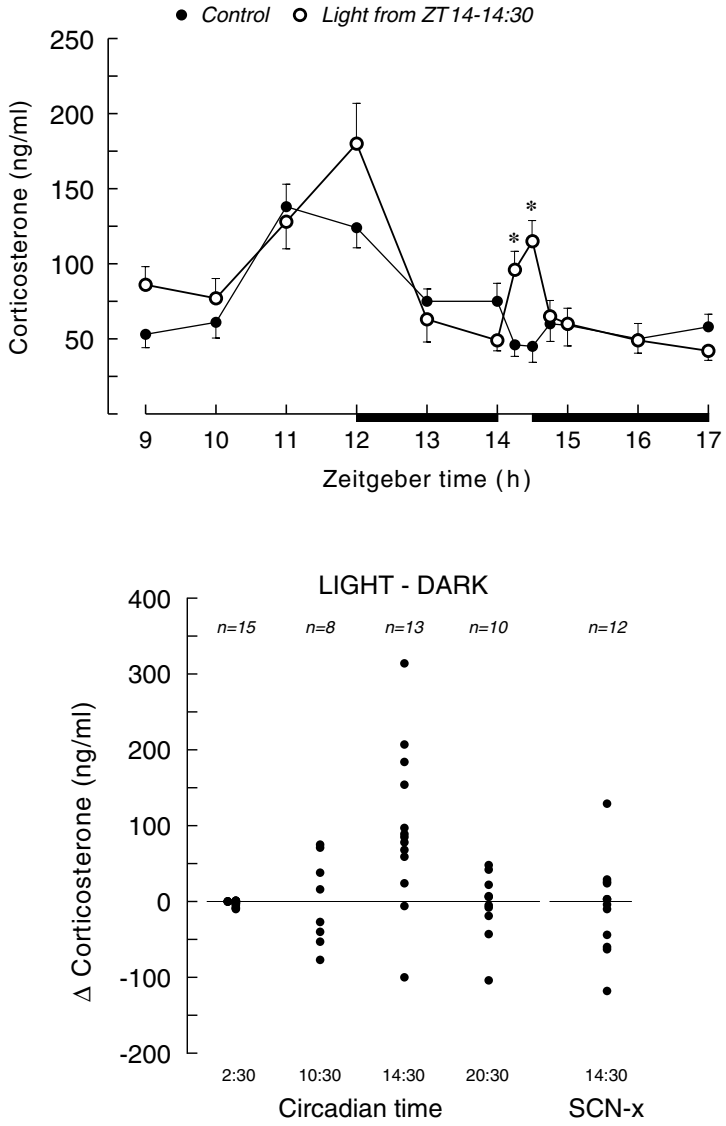


Figure 1 **A.** Plasma corticosterone concentrations (mean ± S.E.M) from CT9-CT17 in animals with (o) or without (●) light exposure at CT14. One-way ANOVA revealed an effect of *time* (p<0.001) in both conditions (light/without light). The two-way ANOVA *light*time* interaction effect indicates that the light exposure, applied at CT14, induces a change in the plasma corticosterone concentrations (* P <0.05 compared to the control group). **B.** Effect of a 30-min light exposure, at t =0 at different Circadian Times, (CT2, CT10, CT14 and CT20) on plasma corticosterone values in intact rats. The right side of the panel shows the effect of light, applied at CT14, on plasma corticosterone values in SCN-lesioned animals.

Table 2 Statistical analysis of (clock) gene expression changes during nocturnal light exposure at CT14 and CT20.

	Per1	Per2	Per3	Cry1	Cry2	Dbp	NAT	MC2R	G6Pase	GK	GLUT2	PY	PEPCK
Pineal													
Time (T)	0.407	<0.001	0.037	0.356	0.035	0.055	<0.001						
Light (L)	<0.001	0.016	0.051	0.854	0.026	0.143	<0.001						
T*L	0.095	0.109	0.069	0.964	0.874	0.016	<0.001						
Adrenal													
Time (T)	<0.001	<0.001	<0.001	<0.001	0.002	<0.001		0.189					
Light (L)	0.478	0.087	0.956	0.258	0.012	0.352		0.256					
T*L	0.673	0.554	0.829	0.617	0.572	0.282		0.035					
Liver													
Time (T)	<0.001	0.009	<0.001	<0.001	<0.001	<0.001			<0.001	<0.001	0.009	<0.001	0.653
Light (L)	0.086	0.357	0.830	0.145	0.241	0.124			0.164	0.003	0.048	0.829	<0.001
T*L	0.011	0.029	0.669	0.781	0.577	0.12			0.155	0.331	0.044	0.133	0.544
Heart													
Time (T)	<0.001	0.334	<0.001	<0.001	<0.001	<0.001							
Light (L)	0.001	0.002	0.346	0.077	0.017	0.314							
T*L	0.070	0.018	0.017	0.775	0.001	0.042							
Muscle													
Time (T)	<0.001	0.147	<0.001	<0.001	0.002	<0.001							
Light (L)	0.409	0.466	0.853	0.031	0.014	0.513							
T*L	0.855	0.310	0.894	0.317	0.473	0.322							

Data were analyzed by a mixed one way ANOVA (Time (2 levels), i.e., CT15 *versus* CT21, or Light (2 levels), i.e., light *versus* no-light). If significant effects were detected, it was followed by a post-hoc LSD test. The differences were considered significant when p values were lower than 0.05.

Table 3 Statistical analysis of (clock) gene expression changes during CT14-15 light exposure in liver-intact and liver-denervated-rats.

	Per1	Per2	Per3	Cry1	Cry2	Dbp	NAT	MC2R	G6Pase	GK	GLUT2	PY	PEPCK
Pineal													
Dener (D)	0.069	0.732	0.086	0.329	0.526	0.019	0.055						
Light (L)	<0.001	0.247	0.909	0.324	0.279	<0.001	0.034						
D*L	0.907	0.930	0.996	0.436	0.255	0.080	0.632						
Adrenal													
Dener (D)	0.729	0.153	0.653	0.297	0.565	0.737		0.008					
Light (L)	0.928	0.055	0.359	0.285	0.651	0.883		0.223					
D*L	0.912	0.677	0.261	0.111	0.261	0.195		0.073					
Liver													
Dener (D)	0.147	0.061	0.810	0.409	0.056	0.595			0.081	0.323	0.116	0.191	0.043
Light (L)	0.087	0.239	0.840	0.138	0.196	0.159			0.808	0.163	0.031	0.033	0.045
D*L	0.046	0.103	0.508	0.564	0.902	0.209			0.850	0.748	0.147	0.617	0.030
Heart													
Dener (D)	0.311	0.038	0.070	0.501	0.315	0.106							
Light (L)	0.552	0.341	0.050	0.663	0.665	0.045							
D*L	0.342	0.197	0.329	0.144	0.304	0.732							

Data were analyzed by a mixed one way ANOVA (Dener (2 levels), i.e., Intact *versus* Denervated, Light (2 levels), i.e., light *versus* no-light). If significant effects were detected, it was followed by a post-hoc LSD test. The differences were considered significant when p values were lower than 0.05.

expression of the MC2 receptor in the adrenal also displays a clear time-dependent change, suggesting a time-dependent change in the sensitivity of the adrenal to ACTH according to the time of the day, as already proposed in previous study [51].

RESPONSE OF THE PINEAL AND THE ADRENAL TO LIGHT EXPOSURE

As shown in Figure 2 A, B, light exposure induced significant changes in the mRNA expression of clock genes in the adrenal and pineal [82, 159]. In the pineal, Per1 expression decreased during both CT14 and CT20 light exposure. Per2 and Per3 expression only decreased during the CT20 exposure, whereas Dbp expression was increased during the CT14 exposure. In addition to these changes in clock gene expression, the expression of NAT, a functional gene involved in melatonin production, shows a pronounced decrease after light exposure at CT20, a time point at which basal NAT is high (Figure 2C).

Although the adrenal, like the pineal, is innervated by the sympathetic branch of the autonomic nervous system, the clock gene expression in this organ displayed a completely different pattern after light exposure. In the adrenal, statistical analysis only indicated a significant increase of Cry2 expression at CT20. On the other hand, the expression of MC2-R, i.e. the ACTH receptor, increased significantly at CT14 but not at CT20 (Figure 2C), matching the corticosterone changes [51, 82].

RESPONSE OF THE LIVER, HEART AND MUSCLE TO THE LIGHT EXPOSURE

In the liver, light exposure caused a significant increase of Per1 and Per2 expression at CT14. The significant *Time * Light* interaction (Table 2) indicates that the Per1 and Per2 responses to light differ according to the time of the night, reinforcing the idea that the light pulse induces a time-dependent effect on these 2 clock genes. No significant changes of clock genes were observed after light exposure applied at CT20 (Figure 2 A, B).

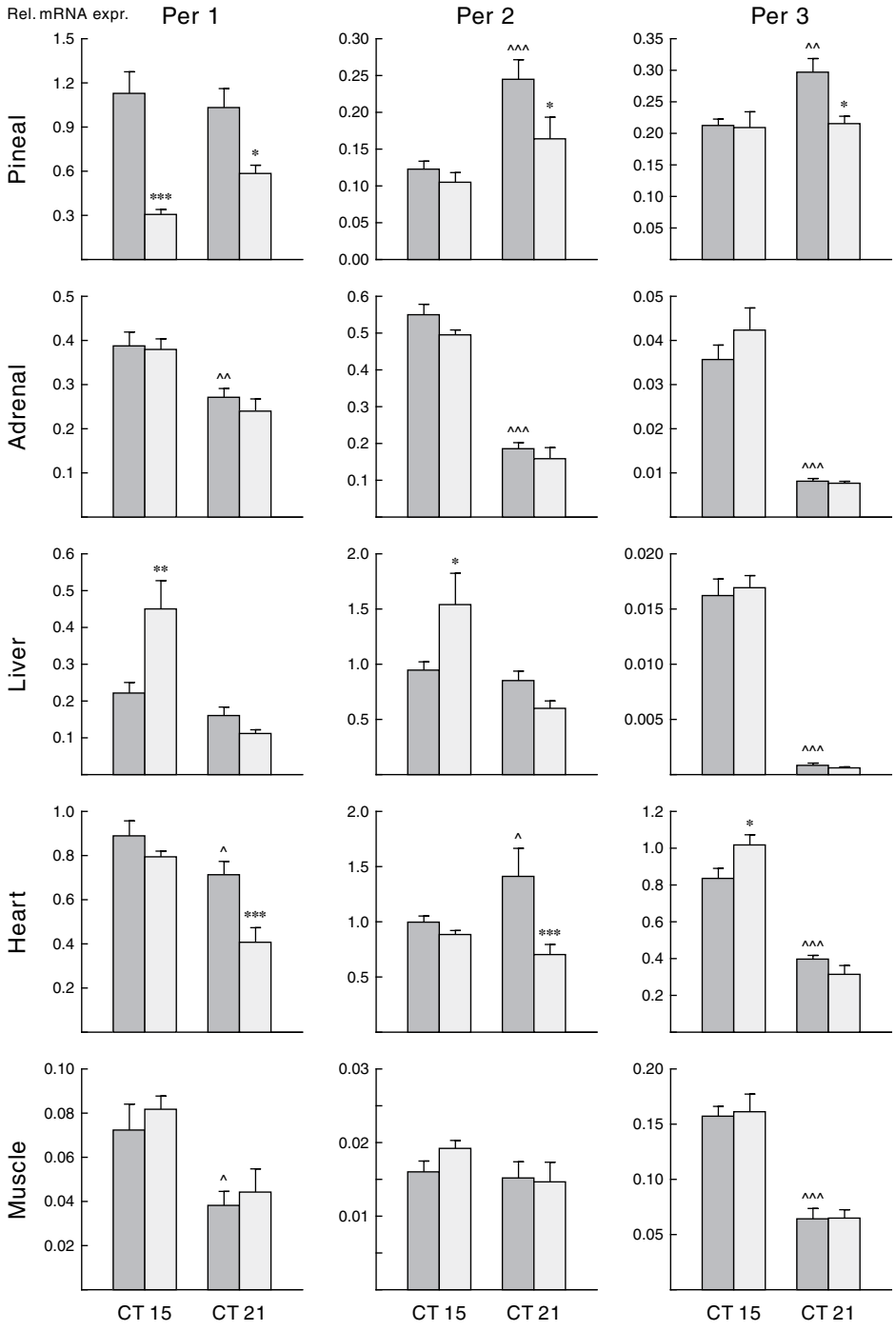
Interestingly, light exposure also induced a change in the expression level of several gluco-regulatory enzymes (Figure 2C). PEPCK expression was increased both at CT14 and CT20, whereas Glut2 expression was increased at CT14. Glucokinase expression decreased at CT20.

In the heart, several clock genes responded to the light exposure. Light applied at CT14 triggered an upregulation of Per3 and Dbp, whereas light applied at CT20 induced a decrease in Per1, Per2 and Cry2 mRNA levels.

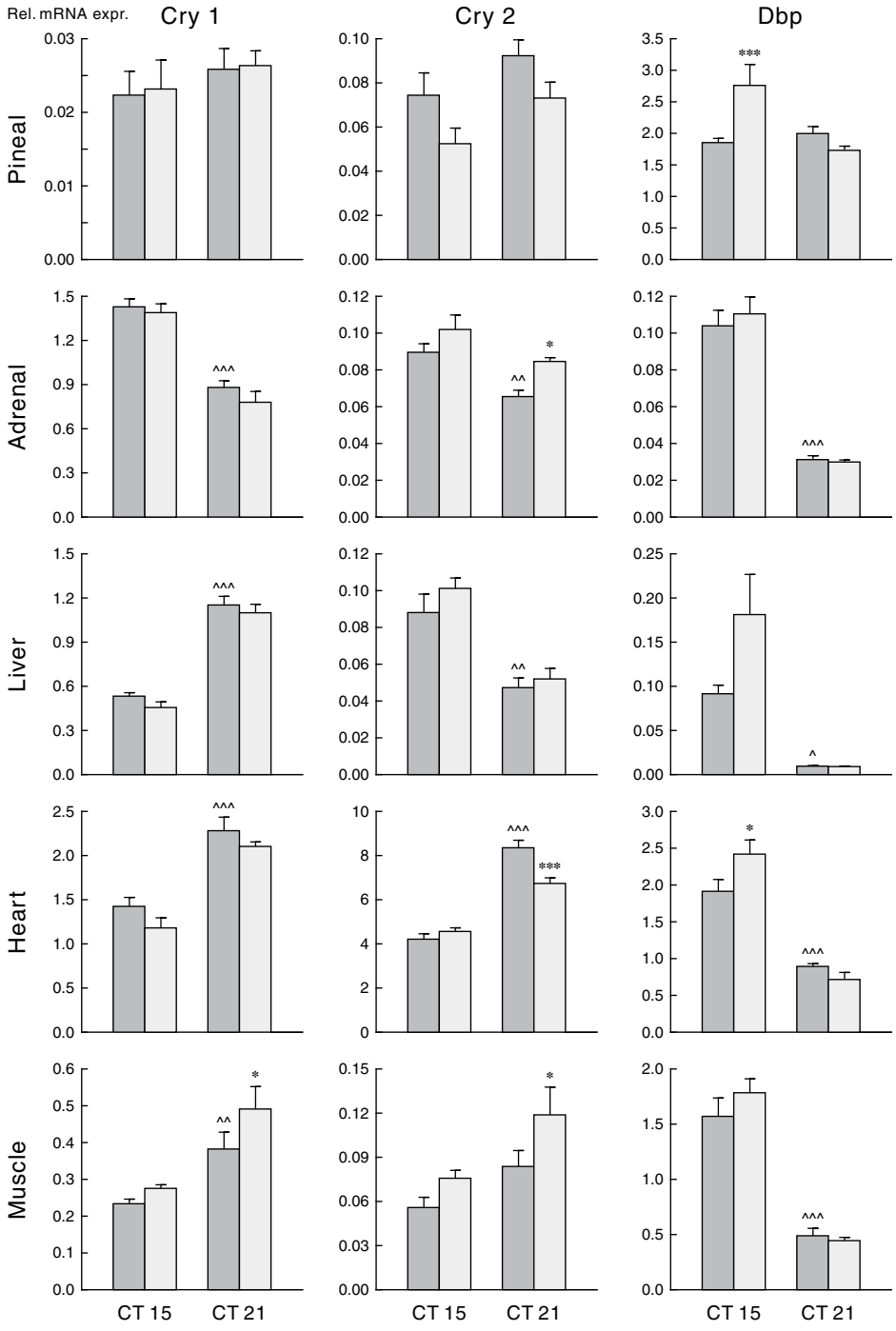
In the muscle, changes in clock gene expression were observed only when light was applied at CT20, when both Cry1 and Cry2 showed an upregulation (Figure 2).

In summary, light exposure affects the expression of clock genes in a differential manner, depending on the organ and on the time of the light exposure. In addition, functional genes in all tissues also showed time-dependent effects of light exposure.

Figure 2 A


Rel. mRNA expr.
Per 1
Per 2
Per 3
Pineal
Adrenal
Liver
Heart
Muscle
CT 15
CT 21

2 B


Rel. mRNA expr.
Cry 1
Cry 2
Dbp
Pineal
Adrenal
Liver
Heart
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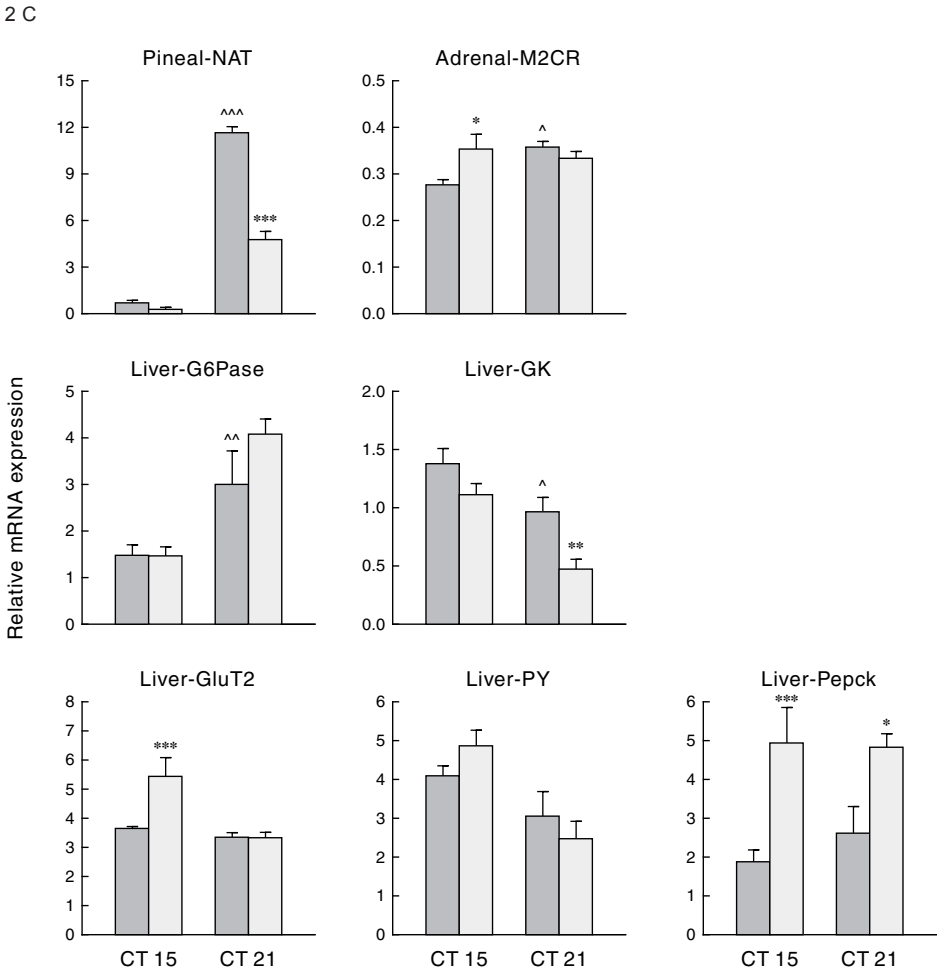


Figure 2 Effects of light exposure at night on mRNA level of Per1/2/3(**A**); Cry1/2 and Dbp (**B**); and enzymes/receptor (**C**) in different organs (pineal, adrenal, liver, heart and muscle). Dark bars represent the non-exposed control group (n = 8 at CT14 and n = 6 at CT20) and white bars represent the light-exposed group (n = 9 at CT14 and n = 6 at CT20). Each value is the mean ± S.E.M (* P<0.05, ** P<0.01, *** P<0.005, compared to the non-exposed control group; ^ P<0.05, ^^ P<0.01, ^^^ P<0.005, compared to the non-exposed control group at CT15).

Effects of light exposure on clock gene expression in animals with a complete liver denervation

To investigate the role of the autonomic nervous system in signalling SCN activity to the liver, we repeated the light exposure experiment at CT14 in a group of CD animals. Ten out of 11 CD rats had a complete denervation. Six out of these 10 CD rats were then subjected to CT14 light exposure and the 4 others had been incorporated in the non-light exposure group.

A complete denervation of the ANS input to the liver had no overall effects on the basal expression levels of clock genes and functional genes. Only in 3 out of 30 combinations did ANOVA indicate a significant effect of denervation, i.e, pineal Dbp, Per2 in the heart and MC2 in the adrenal. However, none of these 3 remained significant in the post-hoc analysis. Therefore, liver denervation per se does not seem to affect the basal expression of the gene studied. On the other hand, in the liver, light-induced changes in mRNA levels of clock genes and functional genes were clearly affected by the removal of the ANS input (Table 3 and Figure 3).

In the liver, hepatic denervation not only prevented the effect of light on clock gene expression (i.e., upregulation of Per1 and Per 2) but also the light changes in PEPCK and GLUT2 mRNA expression. These data suggest that the ANS plays an important role in transmitting light information to the various peripheral tissues.

In the pineal, the CD did not impair the decrease of Per1 expression and the upregulation of Dbp expression after a light exposure at CT14. Interestingly, the effect of light on Dbp upregulation was pronounced in the CD animals. In a similar manner, the inhibitory effect of light on NAT expression appeared to be stronger in CD animals as compared to intact animals (Figure 3 and Table 3).

In the adrenal, the light-induced upregulation of the MC2 receptor was absent in CD animals.

Discussion

The present study demonstrates that nocturnal exposure of an animal to light induces rapid changes in clock gene expression in all five tissues examined. Previous observations have shown that light-induced changes in the activity of the pineal, heart and adrenal all depend on the presence of an intact SCN [51, 52, 82, 178, 269-271]. The present study provides evidence that the observed changes in the liver are induced via the ANS and probably do not depend on changes in the secretion of corticosterone or melatonin. Moreover, the present data show that: 1) light has a differential effect on the clock genes in different organs, and 2) clock gene changes do not correlate with the changes in functional genes. Taking these observations together, it appears that the ´peripheral clock´ does not play a decisive role in the light-response of an organ. Furthermore, 3)

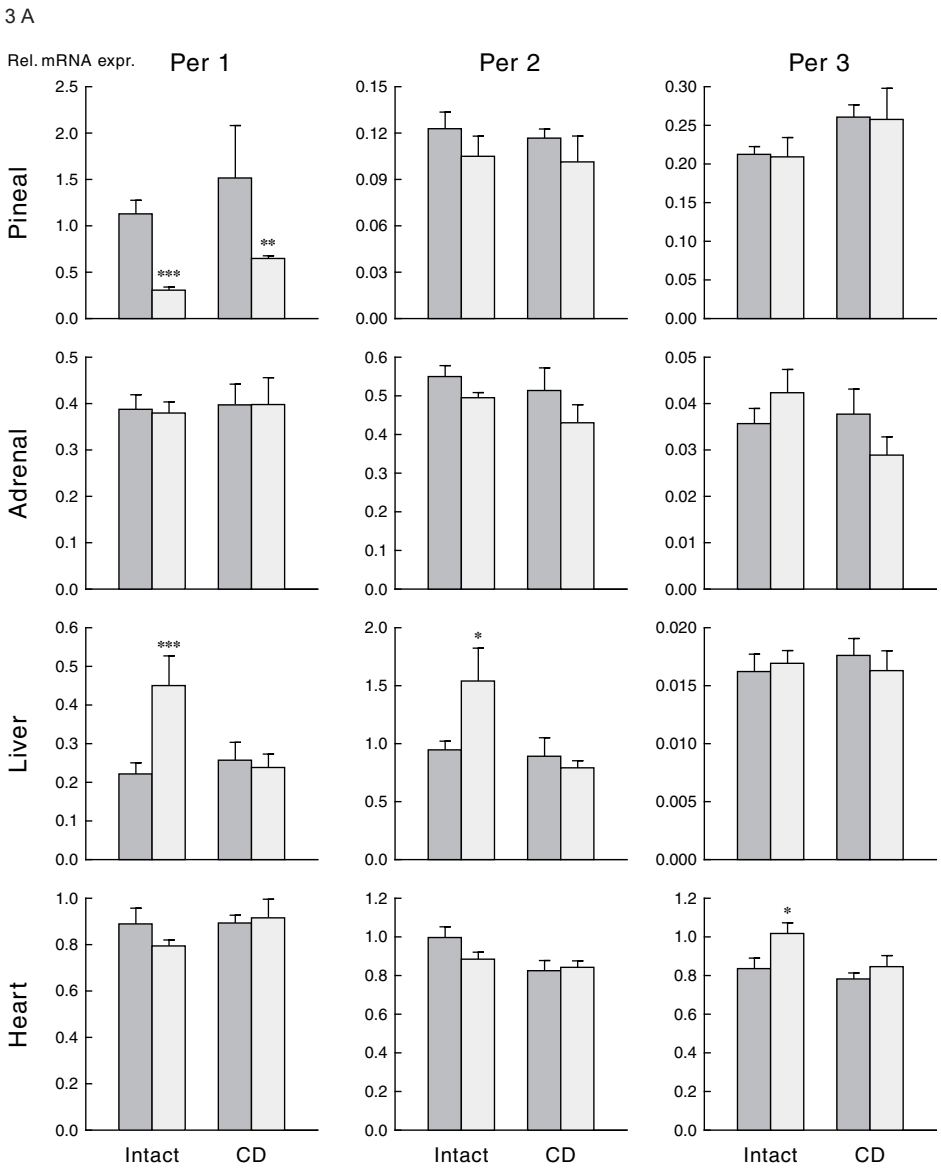
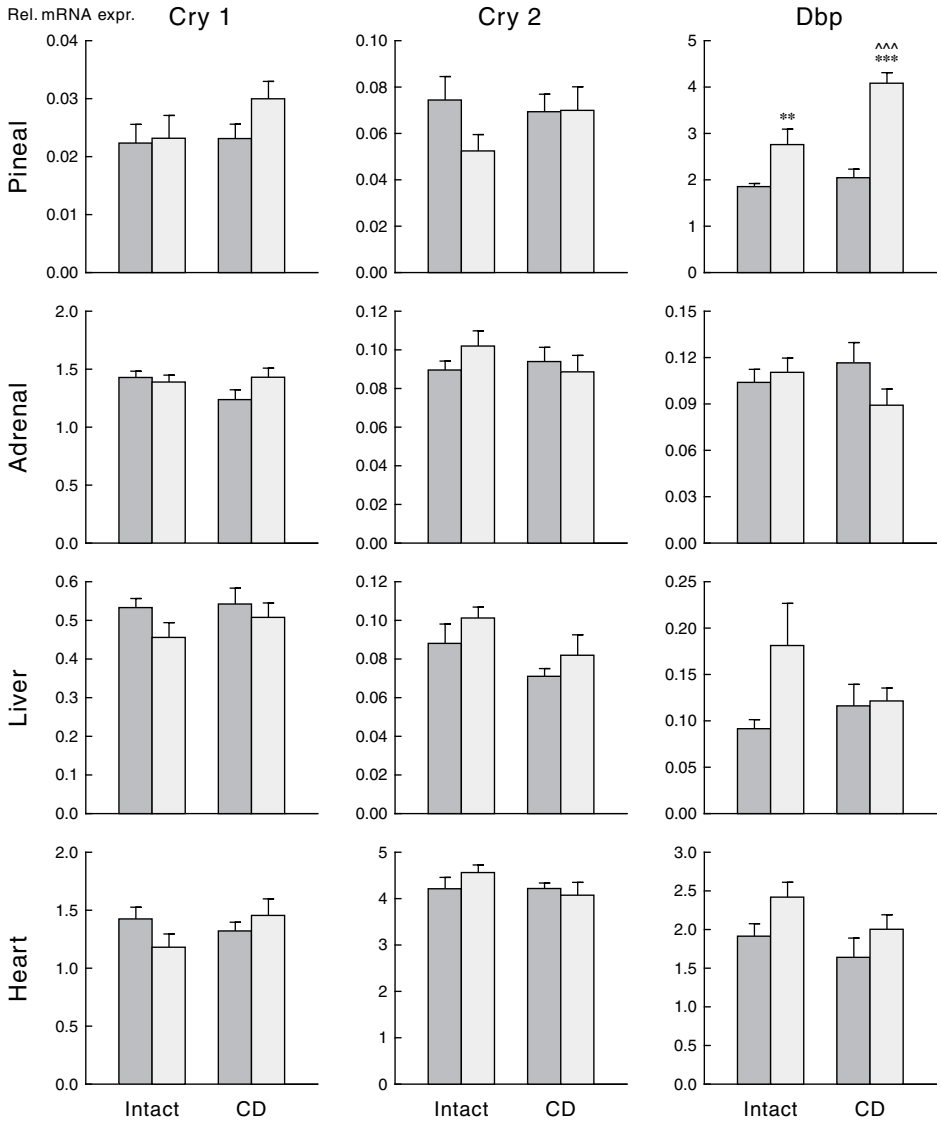


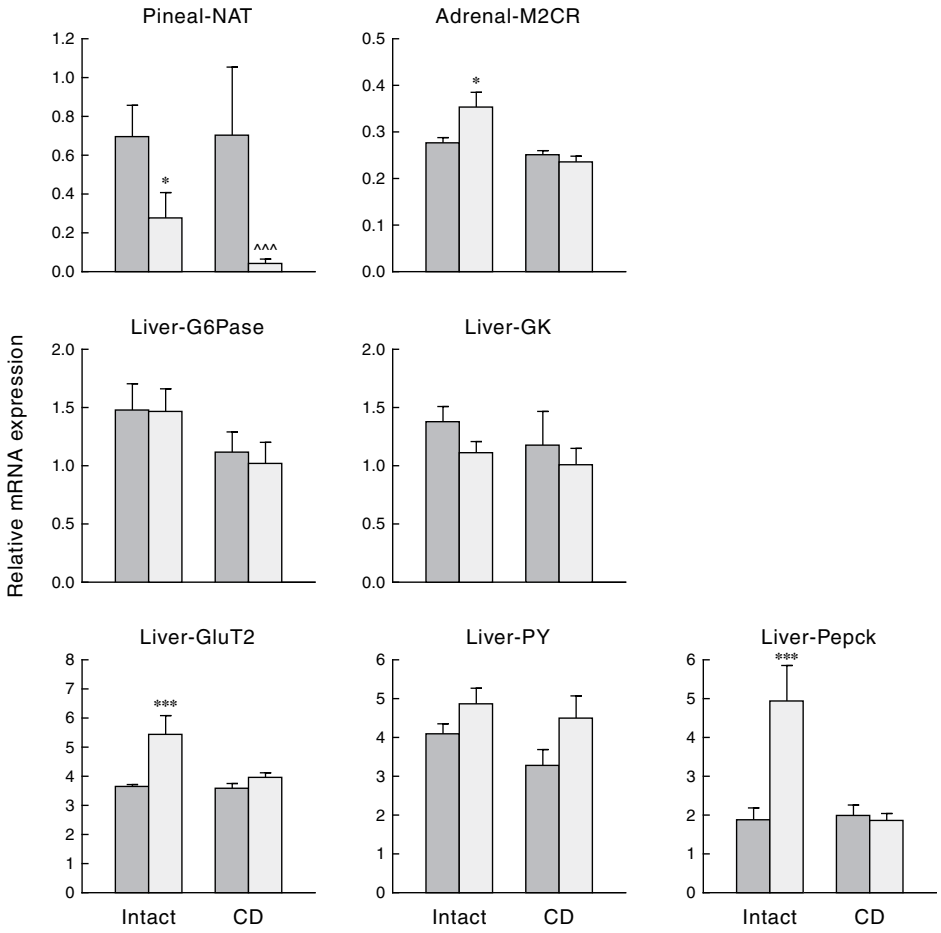
Figure 3 Effects of light exposure at CT14 on the expression of Per1/2/3(**A**); Cry1/2 and Dbp (**B**); and enzymes/receptor (**C**) of different organs in liver-intact (Intact) and liver-denervated (CD) animals. Dark bars represent the non light-exposed animals (Intact, n = 8 and CD, n = 4) and white bars represent the light-exposed animals (Intact, n = 9 and CD, n = 6). Note that the effect of light on Per1, GLUT2 and PEPCK is absent in denervated rats. Each value is the mean ± S.E.M (* P<0.05, ** P<0.01, *** P<0.005, compared to the non-exposed control group; ^ P<0.05, ^^ P<0.01, ^^^ P<0.005, compared to the non-exposed intact group).

3 B



after a complete denervation of the liver, the light-induced changes in this organ are prevented, indicating that the autonomic innervation of the liver is essential for the transmission of the SCN-light information and in this way adjusts the liver metabolism according to the light changes in the environment.

3 C



Role of the ANS in light signalling to the adrenal

The first structure that was recognized to be influenced by light via the SCN and the ANS is the pineal gland. The circadian rhythm of melatonin secretion, the rate limiting enzyme NAT, and the clock genes in the pineal have all been shown to be under the control of the SCN via the sympathetic innervation of the pineal [272]. The Japanese researchers Niijima and Nagai were the first to show the possibility of widespread anatomical connections between the SCN and the peripheral organs [269]. They demonstrated that sympathetic nerve activity to the adrenal, liver and pancreas increased after light stimulation, an effect which was dependent on the presence of an intact SCN, suggesting that the SCN might use the ANS to spread its circadian information to the rest of the body. With regard to the adrenal we previously substantiated this

observation by demonstrating the presence of a multisynaptic pathway between the SCN and the adrenal. The functionality of this connection was demonstrated by the observation of a rapid decrease in corticosterone only after light exposure at CT14. In addition, this decrease was dependent on the presence of the SCN, but occurred without concomitant changes in the release of adrenocorticotropic hormone (ACTH) or the adrenal hormones adrenalin and noradrenalin [51]. Recently this observation was confirmed by Ishida and co-workers, who showed that the increase of plasma corticosterone levels observed after a more prolonged light exposure did not involve the release of ACTH either [82]. ACTH acts on adrenocortical cells and promotes steroidogenesis by specific binding to the ACTH (MC2-R) receptor [273]. This receptor is a G protein coupled receptor (GPCR) that signals to elevate intracellular cyclic AMP (cAMP) levels via adenylate cyclase, similar to NAT [274, 275]. Immunohistochemical staining has shown the presence of MC2-R in the plasma membrane and cytoplasm of the parenchymal cells of the adrenocortical zona fasciculate [276]. Moreover, molecular analysis has indicated that MC2 mRNA is predominantly expressed in the zona fasciculate [277-279]. In the present study we demonstrated that MC-2 mRNA levels were significantly increased after light exposure at CT14, but not at CT20. This observation fits in well with the fact that plasma corticosterone levels were increased at CT14, but not at CT20 (Figure 1), without any noticeable changes in ACTH release.

Earlier studies of Engeland and coworkers [280] have shown that the circadian rise in corticosterone can, at least partly, be ascribed to an increased sensitivity of the adrenal to ACTH. The circadian rise of corticosterone release could only be observed in animals with an intact splanchnic nerve, which also led these authors to propose that the SCN uses the splanchnic nerve to control the sensitivity of the adrenal to ACTH [42, 269]. Our present observation of a simultaneous change in MC2-R and corticosterone suggests that this might indeed be the mechanism by which the SCN affects the daily change in sensitivity of the adrenal to ACTH.

Role of the ANS in SCN-light signalling to peripheral organs

Since light induces changes in adrenal and pineal functionality, resulting in the secretion of corticosterone and a decrease in melatonin production, these two hormones were proposed to be responsible for the light-induced physiological changes throughout body and brain [82, 179, 281]. In the present study, we also observed changes in peripheral clock gene expression in some organs that might be correlated with the light-induced changes in the hormone levels. For instance, in the heart, the upregulation of Dbp and the down-regulation of Per1, Per2 and Cry2 at CT20 might be correlated with the inhibition of melatonin secretion (i.e. decrease of NAT levels). The upregulation of Per3 expression in the heart at CT14 can probably be explained by the increase in corticosterone secretion. These results indicate that the heart may

respond to more than just hormonal cues, depending on the time at which the light is applied. Despite the correlative observation between clock gene and hormonal changes we could draw according to the light-effect on hormonal levels, there is some clear evidence indicating that melatonin and corticosterone are responsible for the light-induced changes in these peripheral organs. Interestingly, in our study we observed that the liver clock genes and functional genes present differential responses which cannot be correlated only with the changes in plasma melatonin or with the changes in corticosterone. In fact, the upregulation of Per1, Per 2 and GLUT2 at CT14 might be correlated to corticosterone secretion, but the changes in Per3 Cry 1 and 2 and of PEPCK can only rely on a different signal. These results suggest that the ANS might participate in the light-induced changes in the liver metabolism as already described for the pineal and the adrenal. Indeed, we have shown that the effect of light at CT14, on Per1 and Per2, but also on the liver enzyme expression GLUT2 and PEPCK, was completely abolished after complete denervation of the liver, indicating that in the liver the light effect is not only mediated by the hormonal cues but via the ANS.

However, the light-induced increase in adrenal MC2-R did not occur in CD animals, which might indicate a change in the light-response of the adrenal (i.e., corticosterone secretion). Therefore, we will need to investigate whether the absence of an MC2 increase in the adrenal in these animals also means that corticosterone is not elevated by light in liver-denervated animals. However, this possibility seems unlikely since no change in the pineal-response to light was observed in the CD animals either.

Role of the ANS in the SCN-light signalling to the liver

As mentioned above, we clearly showed that light affects liver metabolism through the autonomic input and not via light-induced corticosterone secretion. The liver has a major role in energy metabolism, a process which also shows a prominent circadian regulation and requires intense autonomic interaction with the brain. The lack of light-effect on the MC2-R expression in CD animals might also indicate that interfering with the autonomic innervation of the liver disturbs the feedback from the liver to the brain, which in turn may result in a disturbed autonomic output. In fact, it has recently been observed that a dysfunction of the liver function (i.e., lipid metabolism) influences peripheral organs, such as adipose tissue, via the vagal afferent nerve [282]. Previously we have shown that the SCN controls plasma glucose concentrations via the sympathetic innervation to the liver [73]. Our present observation that light can also affect the expression level of certain gluco-regulatory enzymes in the liver illustrates the sensitivity of our peripheral organs, and in this case the liver, for autonomic input and hence for the output of the SCN. In addition, this is further evidence that important changes in liver functionality can be induced by the SCN and are affected via the ANS.

Peripheral clock genes: the same molecular mechanism as within the SCN?

The molecular mechanism of the SCN clock function is proposed to be composed of several rhythmically expressed genes, consisting of interacting positive and negative transcription/translation feedback loops with an approximate period of 24 hours [25, 283, 284]. Besides the SCN, sets of clock genes are also rhythmically expressed in peripheral tissues, suggesting a possible universal role of clock genes to mediate the temporal control over physiological circadian events in peripheral organs. In this respect it is interesting to note that the relative amounts of the clock genes show enormous differences among the different tissues studied. For example, at CT15 the relative expression of Per3 in pineal, adrenal, liver, heart and muscle varies from 0.015 - 0.800, i.e., more than 50-fold.

Light affects clock gene expression in a differential manner, depending on the organ: i.e., light decreases Per1 expression in the pineal, whereas Per1 expression is increased in the liver after light exposure at CT14. Surprisingly, although the pineal and the adrenal are both sympathetically innervated also in these tissues, the clock gene response to light shows a completely different pattern, i.e., a decrease in pineal Per1 and increased Dbp expression at CT14, but no change in the adrenal. Alternatively, a decrease occurs at CT21 in Per1 and Per2 in the pineal, but again, there is no change in the adrenal. In our opinion these observations indicate that the functional relationship between clock genes differs, depending on the organ. Furthermore, the fact that light affects clock gene expression in a differential manner, depending on the time of day and on the organ, supports the notion that the clock gene mechanism and its function in the peripheral organs is not universal, and is far from being understood completely. Consequently, this suggests that also the interaction between the clock genes in the different organs is unlike the mechanism in the SCN and may change from organ to organ. The fact that Ishida and coworkers [82], using a similar light stimulus in mice, found a strong increase in Per2 in the adrenal and not in the liver, while in the present study we observed no changes in Per2 but did find changes in Cry 2, both in liver and adrenal, only emphasizes the variability of the responses of clock genes in the organs. Clearly this is an observation not yet encountered in the SCN, where the responses, also from species to species, are remarkable similar.

Furthermore, the mechanism via which corticosterone affects the transcription of Per in the liver is not known. However, it seems logical that the mechanism of corticosterone binding and its coupling to the clock genes will be the same in all tissues. After a light exposure at CT14, which induced a secretion of corticosterone, we observed no changes of Per1 expression in the heart and in the muscle, whereas in the liver Per1 gene was up-regulated. These data indicate that the action of corticosterone on clock gene transcription is not so widespread as is sometimes suggested, and other types of signals might be involved in the light-induced physiological responses, according to the organ.

The function of clock genes in the periphery has been a topic of much research, but to date only limited evidence for their function is available. Clock genes are involved in cell division [285-287], circadian locomotor rhythms [86], heme metabolism [288, 289], bone metabolism [157] and energy homeostasis [290]. In the present study we have shown that light induces functional changes in liver and adrenal but that these changes have no correlation with the changes in the clock genes. In the pineal, in agreement with other studies, light-induced functional changes were found as well (NAT expression); it is not clear whether these changes bear any relation with the changes in clock genes. In addition, although some clock genes and NAT in the pineal may share the same regulatory pathway, i.e. the multisynaptic pathway between SCN and pineal [159, 291], no causal relation has been shown between them in this study nor in previous ones [292]. Also, the delayed and different responses of clock genes in peripheral organs after light exposure suggest that some peripheral functions can be controlled by the SCN without the direct involvement of the peripheral clock genes (at least in as far as can be seen from the mRNA level). In recent years, several studies demonstrated that, besides light information, also other cues, such as restricted feeding [168], chemicals [175, 177], and temperature [293] may act as entraining signals for clock genes. We speculate that whereas the central clock genes in the SCN have a clear function in controlling the rhythmic activity of SCN neurons, peripheral clock genes probably lost this main function once the central autonomous clock had evolved. Consequently, unlike the clock genes in the SCN, clock genes in the periphery may respond to many more factors than just light, but these have retained only a very tissue-specific (and probably very limited) functional significance.

In conclusion, the present results emphasize that light-induced changes in SCN activity and output not only result in immediate changes in the pineal and adrenal, but also affect the physiology of the heart and liver. Moreover, the SCN may use multiple pathways to transmit these light-induced changes to the rest of the body, but direct signalling via the ANS plays an essential role, which enables the SCN to target its message to specific organs.

Acknowledgements

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Chapter 6

A discussion on the role of peripheral clock genes and the suprachiasmatic nucleus in the rhythmic functions of organs

Biological clock mechanisms play an essential role in adapting and synchronizing an organisms' physiology and behaviour with the environmental light/dark changes [25, 167, 258]. But, maybe even more importantly, clock mechanisms allow the organism to anticipate these changes [65, 201]. Because of the everlasting influence of sunrise and sunset, these clock mechanisms have developed in all organisms, including the most primitive unicellular ones. In mammals, this biological or circadian clock is located in the suprachiasmatic nucleus (SCN), which is situated just above the optic chiasm, and receives direct light information via the retinohypothalamic tract. The pacemaker property of the SCN relies on a molecular mechanism that is based on interacting positive and negative transcriptional translational feedback loops [24, 25, 193]. The integrity of the molecular clockwork is essential for the functionality of the SCN to generate daily rhythms in physiology and behaviour and to adapt these functions to the environmental changes [55, 87, 294, 295]. In the early nineties it was discovered that in *drosophila*, but also in vertebrates such as zebrafish, clock mechanisms were present in all tissues [196, 296-299]. A few years ago the components of the molecular clockwork (called clock-genes) were found, also in mammals, not to be restricted to the SCN, but located throughout the entire body, in agreement with the evolutionary origin of the clock mechanism. In more complex organisms the vital clock functions have moved to the central nervous system. Thus the mammalian circadian timing system is now considered as a hierarchical organization in which the SCN is the master clock, which entrains and synchronizes the peripheral oscillators [199]. Besides the presence of clock genes in peripheral tissues, oscillatory patterns of genes involved in metabolic, apoptotic or mitotic functions have been observed in peripheral organs as well [161, 166, 228, 300, 301], suggesting that the peripheral clock may not just be an evolutionary relic, but may also contribute to organ-specific rhythmic output [244, 302, 303].

Since then many studies have been performed to elucidate how the SCN coordinates the peripheral oscillators and, especially, whether in mammals these peripheral clock genes drive rhythmic gene expression within the peripheral tissues, resulting in an organ specific function. Until now, a clear role of the peripheral clock genes has been reported, in mammals, only at the cellular level, i.e., the control of cell division [156, 157, 216, 304, 305], of natural killer cell functions [306], of adipogenesis [307], and the circadian control of the gene expression of some key enzymes involved in metabolism, such as steroid 15 a-hyproxylase (Cyp2a4) [308]. However, as, to date, no clear evidence has been presented

in mammals for a role of the clock genes in the regulation of the metabolic function of a whole organ, i.e. circadian control of rhythmic output (Chapter 2). Moreover, the question how the SCN sends its timing signal to the periphery in order to influence the metabolic activity of an organ according to the time of day is still not completely clear. In the first part of this general discussion we will focus on the role of the clock genes in organ-specific output and in the second part we will discuss the role of the autonomic nervous system (ANS) in the transmission of the SCN signal to the periphery.

Role of the peripheral clock to execute SCN signaling

Peripheral clock genes: a left-over of evolution?

As mentioned above, studies performed in mammals reported a direct link between clock genes and peripheral cell function, i.e., the control of Cyclin Cdc2, a key regulator of mitosis [216, 268], indicates the circadian control of cell division (e.g. osteoblast growth [157]). A similar function of clock genes is also reported in Neurospora and Zebrafish [309, 310], indicating an ancestral function maintained throughout evolution. Interestingly, it is only in invertebrates or lower vertebrates that peripheral oscillators exhibit a role in physiological functions such as, eclosion or the olfactory system [311-314], suggesting a difference between lower and higher organisms in the "use" of the peripheral clock genes. This discrepancy between species has been extensively studied and discussed in terms of the analysis of the molecular mechanism of the clockwork [196, 315], but it might also be that the role of clock genes within mammalian peripheral tissues is not as essential for physiological functions as for *Drosophila* or Zebrafish [275-278]. Indeed, both of these species have peripheral oscillators that are directly sensitive to, and entrained by, light [297, 316, 317]. In mammals, the retina is the only structure containing clock cells that receive direct light input. Within the SCN only a limited number of neurons receive light information from the retina, which is then spread by the SCN to the rest of the body in order to synchronize physiology and behaviour to the L/D cycle. When the SCN is lacking, the peripheral oscillators exhibit an external and internal asynchrony, reinforcing the idea that, in the course of evolution, the organization of the circadian timing system moved from the periphery to the central nervous system (the SCN) and that clock mechanisms in our peripheral organs are not directly sensitive to light. Possibly this is one of the reasons why until now no essential rhythmic functions for clock genes at the organ level have been demonstrated and why the functions that have been found are limited to the cellular level, e.g. associated with cell division and cell growth.

Striking observations on peripheral clocks in mammals

In view of the proposal that peripheral clocks may regulate organ function [65, 82, 198, 318], it is quite surprising to observe a strong difference in the basal levels of circadian

clock gene expression between different organs. Per1 mRNA levels, for instance, are much higher in the heart than in the liver (see Chapter 5). Other studies have reported similar differences between the kidney and testis [163, 319, 320], and also between different brain areas [321]. But, more surprisingly, although Per1 is expressed at a higher level in the heart than in the liver, Per2 expression is identical in both organs, indicating a differential expression of relative clock gene levels among organs as well. Taking all this together, the question arises what molecular mechanism underlies the clockwork in each tissue, as well as the question of the functional significance of the clock genes for rhythmic functions of the organ.

In view of the complex circadian timing system, which adapts several physiological parameters to the environmental changes, the question is why some tissues, such as testis or thymus, do not display a rhythmic expression of clock genes [160, 163]. As it was demonstrated that cell proliferation is controlled by clock genes, it is remarkable that especially these two organs which fulfil a comparable role in cell function (spermatogenesis or T cell production, respectively), do not exhibit a clock gene rhythmicity (i.e. mechanism) similar to the epithelial cells or the bone marrow [156, 322].

Moreover, in our experiment, in which we applied light at two CT points (CT14 and CT20) and investigated not only how the SCN could signal its message to the periphery but also the role of clock genes in the physiological output of peripheral organs, we observed a discrepancy between clock gene responses and organ-output. For instance, in the pineal, the light response of Dbp (clock-controlled output gene) differs for CT14 and CT20, whereas at both time points the AA-NAT (aryl akylamine N acetyltransferase, i.e., the enzyme involved in melatonin production) expression is decreased. In the liver, too, light induces an increase of PEPCK expression at both time points, whereas Per1 expression is up-regulated only at CT14. These observations led us to reconsider the idea that clock genes are involved in the circadian physiological response of an organ, and to consider the possibility that the clock genes may have a much more limited function.

Liver oscillator: Role in rhythmic glucose metabolism?

In view of the role of clock genes in rhythmic-organ output, we investigated whether the liver clock genes play a role in the genesis of the rhythmic glucose output of the liver. Indeed, we found that the daily plasma glucose rhythm is affected by the same type of signalling as the liver clock genes, i.e., food intake, hormonal cues, such as corticosterone, and autonomic nervous system (Chapter 2/3) [79, 92, 168, 171, 180, 181]. In our set of experiments, however, we demonstrated that food intake is not the main Zeitgeber for the liver oscillator (Chapter 2), nor for the glucose rhythm [92]. On the other hand, in HSx animals in which the glucose rhythm is abolished, the rhythmic pattern in liver clock genes was not affected (Chapter 2), which was the first indication that liver clock genes

might not be essential for the hepatic glucose metabolism. As a second indication, we observed in these HSx animals an abnormal daily profile of enzymes involved in glucose metabolism (e.g., G6Pase, GK, PEPCK). Some even displayed a complete absence of daily rhythms, whereas the clock gene expression remained unchanged (Chapter 2/3). Moreover, in our experiments, in which we aimed to identify the influence of the ANS and food intake on liver enzymes, we found that the SCN uses different pathways to control the circadian profile of the liver enzymes, i.e. the PEPCK rhythm is regulated via the ANS, GK and G6Pase are influenced via feeding activity, and the glycogen rhythm seems to be controlled via hormonal signals (likely by corticosterone) (Chapter 3). Most interestingly, in spite of the change in liver enzymes rhythmicity, the daily expression of the liver clock genes remained unchanged in all experimental conditions, reinforcing the idea that the liver clock gene are not involved in the oscillatory pattern of the liver enzyme (Figure 1 and 2). All these data strongly suggest that the liver clock genes are not involved in controlling the circadian profiles of the liver enzymes studied and, consequently, are not involved in the rhythmicity of hepatic glucose output.

Interestingly studies performed in Clock KO mice reported that these mice show a tendency towards obesity and metabolic syndrome, suggesting an important role of the circadian clock gene, or at least the clock, in the energy balance [323, 324]. However, in such a KO model the circadian system of the entire organism is disrupted.
This total disruption also leads to abnormal food intake and locomotor activity patterns, making it difficult to identify whether the metabolic disturbance is a direct or indirect consequence of the disturbed peripheral clocks. In addition, the value of this study is much debated because other groups, using another background for the clock mutation, did not observe obesity as a consequence of this disturbance (personal communication of Ruud Buijs).

Recently, an elegant study investigated the role of clock genes in the heart by performing a organ-specific disturbance of the clockwork (i.e., over-expression of Clock, a component of the positive feedback loop) in this organ [325]. Using this molecular tool, the authors succeeded in demonstrating a role of the clock genes in the cellular lipid metabolism (i.e., cardiomyocytes). This recent finding might explain how an impairment of the clock mechanism within a tissue may lead to organ pathology, such as a fatty heart, or even cancer [325, 326]. Therefore, disturbance of specific clock genes in a specific organ might prove a better approach to investigate the role of clock genes in tissue functionality. But, despite this cellular function of the peripheral clocks, there is as yet no clear evidence of their role in the entire-organ output.

A common signalling pathway

In the present thesis we used a light pulse at night to investigate how the SCN perceives such an environmental change and how it transmits this information to the peripheral

organs so that they can adapt the necessary physiological parameters. Previously light-induced physiological effects had been shown in the pineal (inhibition of NAT and then melatonin secretion [81, 159, 327-329]), in the adrenal (firstly corticosterone secretion is inhibited, then stimulated [51, 82]), and in the heart (heart rate is decreased [52]). In a similar way, recently Ishida and coworkers reported a positive correlation between clock-gene expression and hormonal responses, i.e., corticosterone secretion, suggesting a potential role of clock genes in the timely re-adjustment of peripheral organs. However, it is unknown which cellular signalling pathway connects the clock genes to the metabolic enzymes involved in the hormonal response. In line with this it is not clear why the light-induced clock gene responses differ whereas the physiological response remains the same (e.g. pineal) (Chapter 5). This does not seem to be in line with an essential role of the clock genes in such a physiological function. Moreover, components of the clockwork may be affected by proteins involved in cellular signalling pathways, such as Ras/mitogen-activated protein kinase (MAPK), calcium/calmodulin-dependant kinase (CaMK)II [330], but also by components essential for the cellular metabolism, such as nicotinamide adenine dinucleotide (NAD) [331]. This suggests that the peripheral oscillator responds to similar signals as other cellular components involved in cell function. The correlative data obtained from clock genes and physiological responses indicate that they are induced by the same signalling pathways, but obviously this does not mean that clock genes generate the physiological response. In line with this idea, a recent study reported the existence of multiple promoters on peripheral clock genes (in contrast to those in the SCN), indicating the capacity of these components of the oscillator to respond to multiple signals [207].

In summary, there is ample evidence that the pacemaker located in the SCN differs from the one(s) in peripheral tissues, both in terms of molecular mechanism as well as with respect to their responsiveness to the environment. Although it has been widely accepted that the functionality of the SCN clockwork is required to generate a rhythmic output, the evidence for for a role of the peripheral clock genes in the genesis of an organ-specific output appears to be much less obvious. Therefore, we would like to propose that in mammals peripheral oscillators do not play an essential role at the organ-level, but rather at the cellular level (i.e. associated with cell division and cell growth).

Role of the Autonomic Nervous System in signalling the SCN message to the periphery

The SCN uses multiple pathways to send its timing signal to the peripheral organs: neural routes (the two branches of the ANS) and hormonal routes (e.g., melatonin and corticosterone) [65, 71]. For instance, the rhythmic activity of the pineal (involved in the secretion of the melatonin) is regulated by the sympathetic innervation, but the

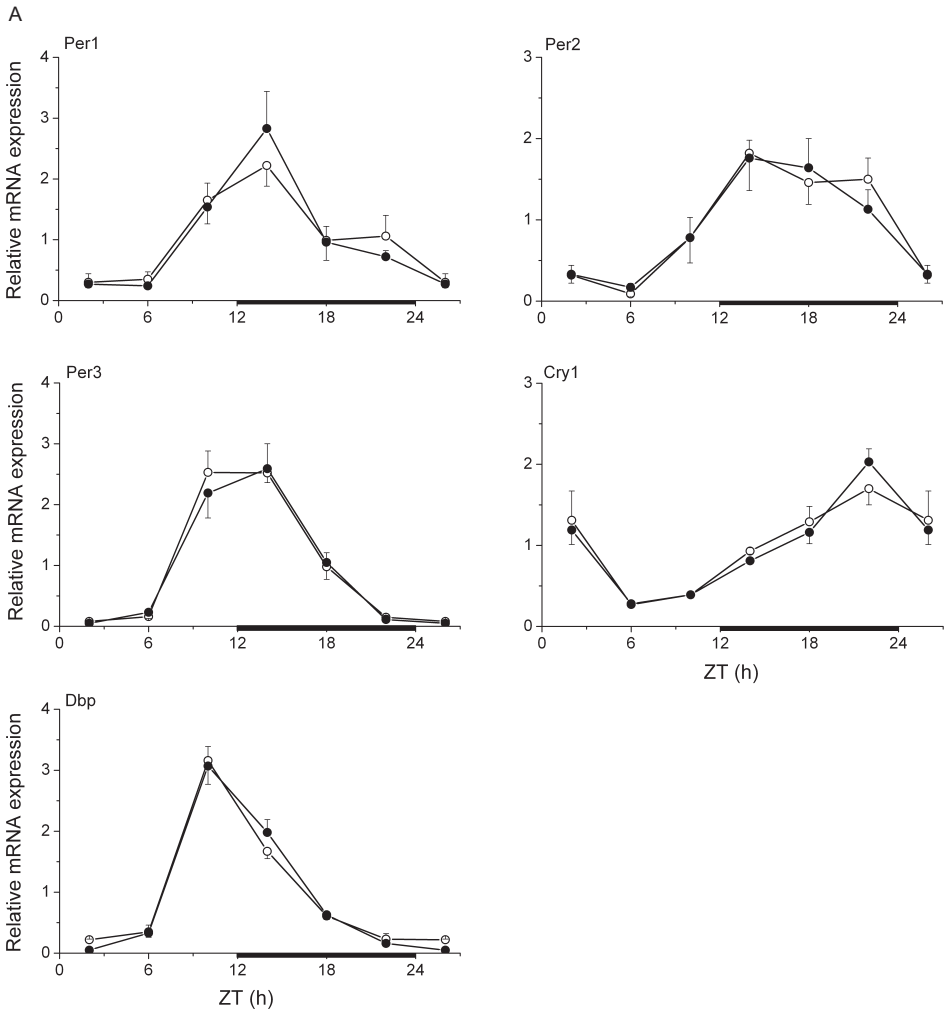
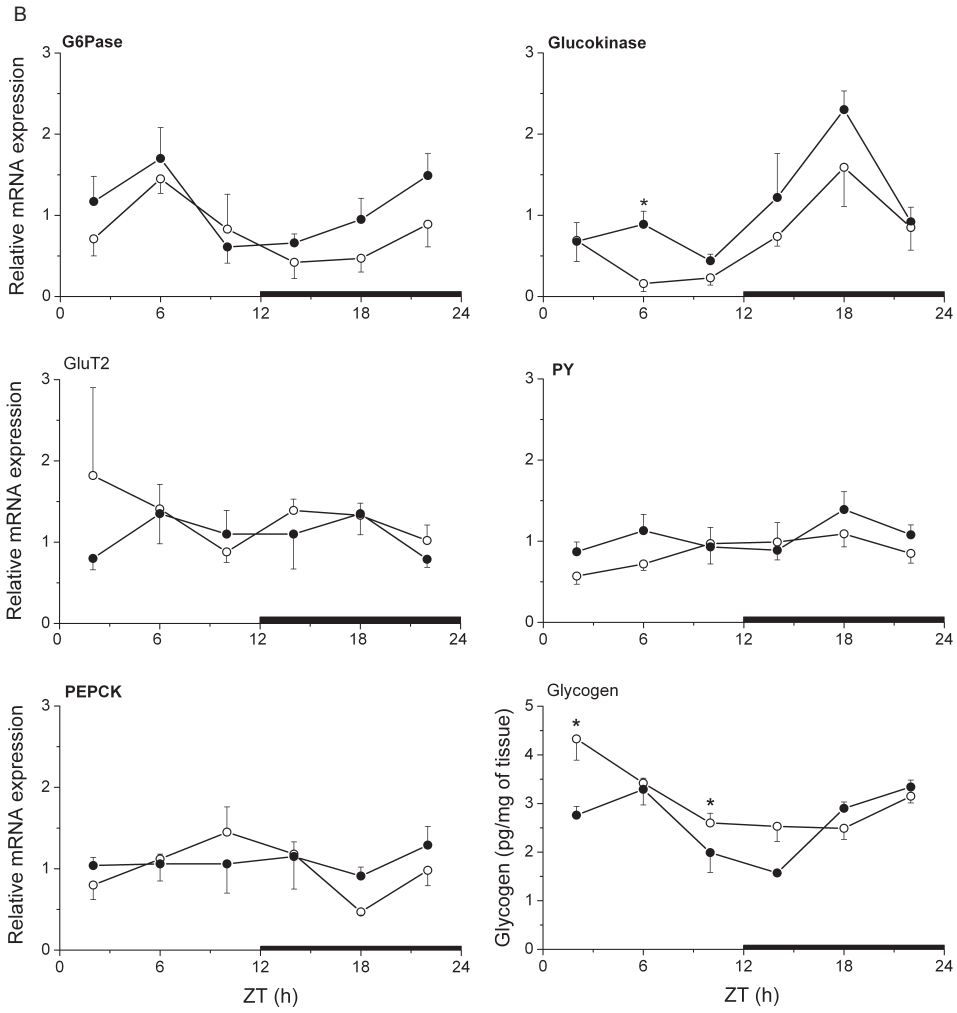


Figure 1 **A.** Daily profile of the clock gene mRNA expression along the L/D in sham-operated (o) and CD (●) animals fed *ad libitum* (same group of animals as presented in B). **B.** Daily profile of the liver enzyme mRNA expression along the L/D cycle in sham-operated/CD animals fed *ad libitum* (Chapter 3).

rhythmicity of the adrenal (which generates the 24h-rhythm in corticosterone, but also a surge of corticosterone in case of stress) is regulated by its sympathetic input as well as by the pituitary hormone ACTH. The heart receives inputs of both ANS branches but also responds to adrenaline and thyroid hormones, whereas the liver (which is responsible for the glucose/lipid metabolism, but has many more functions as well e.g. detoxification) is innervated by both autonomic nerves and responds to a multitude of hormones/nutrients present in the blood (e.g., insulin, glucagon, corticosterone,



melatonin, free fatty acids). Interestingly, it seems that there are a great number of mechanisms that the SCN might use to impose its timing signal to an organ, the exact mechanism depending on the physiological function of the organ. Recent studies have shown that different neurons in the SCN have a different functionality and can drive either sympathetic or parasympathetic outputs, but in addition, different neurons can also target different organs [75, 76, 155]. At present we assume that this differentiation within the SCN is the anatomical basis for the different patterns in hormone secretion [155]. Our studies on the 24-h rhythm in plasma glucose levels have revealed that the SCN indeed uses multiple mechanisms to control the hepatic glucose output in a circadian manner, i.e., ANS, food intake and hormonal cues (likely corticosterone), which all have a specific action on liver physiology and metabolic enzymes (Chapter 3).

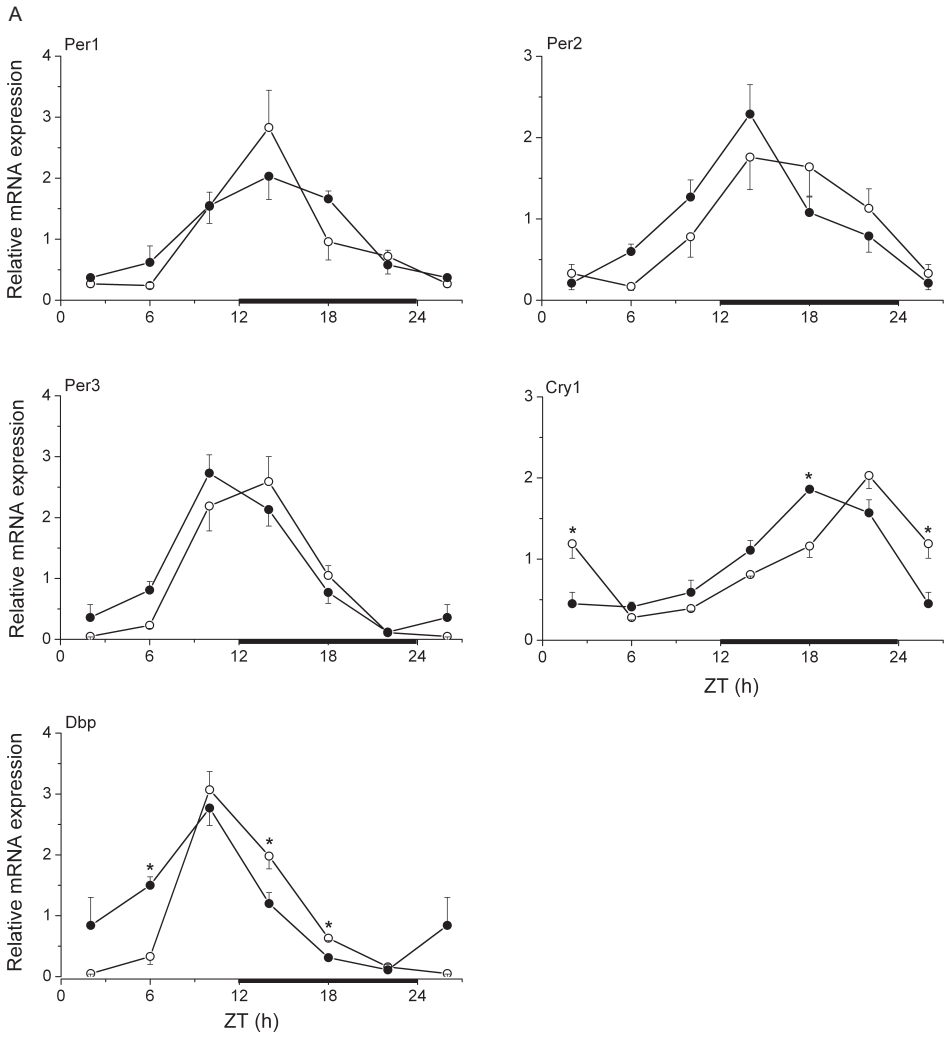
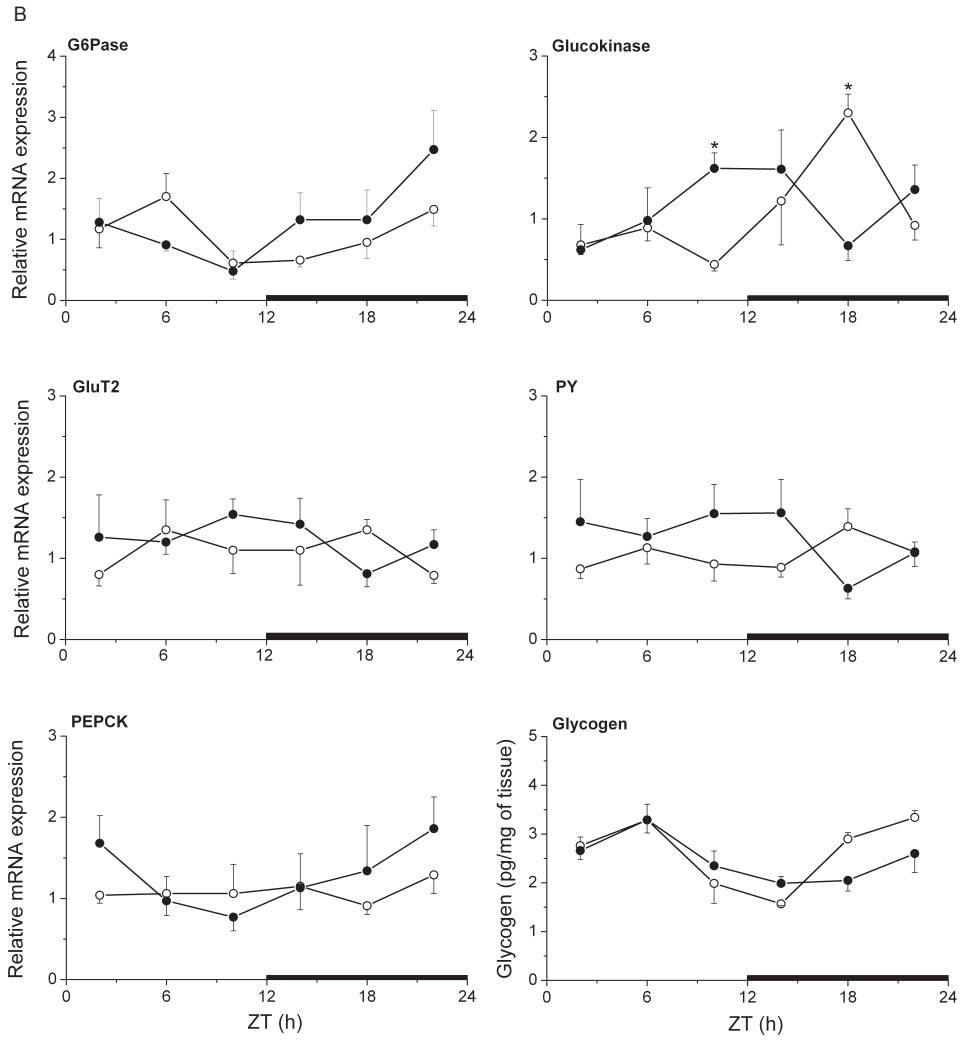


Figure 2 **A**. Daily profile of liver clock gene mRNA expression along the L/D cycle in CD animals fed *ad libitum* (○) or subjected to 6 meals schedule (●) (same group of animals as presented in B). **B**. Daily profiles of the liver enzyme mRNA expression along the L/D cycle in CD animals fed *ad libitum* or subjected to scheduled feeding regimen (Chapter 3).

Dysfunction of the ANS

In the present thesis, we demonstrated that a dysbalance of the autonomic input to the liver (induced by the removal of one autonomic branch), induces an impairment of liver function and impairs glucose metabolism (Chapters 2, 3 and 4). We show that this autonomic dysbalance, created by removing the sympathetic innervation of the liver, directly affects enzymes involved in hepatic glucose production. Interestingly,



one of the symptoms of diabetes type II is an impairment of the HPG control [332, 333], suggesting that a dysfunction of the ANS might be the mechanism responsible for liver disturbance in this metabolic disease. In line with our hypothesis, many studies have reported a dysfunction of the ANS in metabolic diseases, such as diabetes type II, metabolic syndrome and obesity [233-236, 334, 335]. Since glucose and lipid metabolism are tightly regulated by the liver [127], it is not surprising that a dysbalance of the autonomic input to the liver might also disrupt hepatic lipid metabolism, which is another symptom associated with metabolic diseases, such as obesity and the metabolic syndrome. Retrograde tracing studies have shown that at the level of the brain stem (in the DMV), and even in the hypothalamus in the PVN and SCN, the liver and intra-abdominal fat

share the same (pre)autonomic neurons [76], which suggests a neuronal link between glucose uptake in the liver and in intra-abdominal adipose tissue. Recently, Uno and co-workers provided additional physiological evidence that feedback information from the liver may affect adipose tissue via the ANS, indicating another way of cross-talk between these two organs [282]. Taken all together, it might be interesting to use this "hemi-denervation model" to investigate further whether an unbalanced autonomic outflow to the liver, as well as the disruption of hepatic glucose metabolism, might affect the function of peripheral organs.

Role of the SCN in autonomic dysbalance

As mentioned previously, the SCN plays an important role in the coordination of physiological and behavioural parameters according to the L/D or sleep/wake cycle. The SCN uses two main routes to communicate with the periphery: hormonal routes and neuronal routes (i.e., the two branches of the ANS). Retrograde tracing studies revealed separate populations of parasympathetic/sympathetic (pre)autonomic neurons, not only at the level of the PVN but also within the SCN [155], suggesting that the SCN is able to control, differentially, the two branches of the ANS. In line with this hypothesis, the antagonistic roles of the autonomic input to the liver (i.e., sympathetic activity stimulates glucose output whereas parasympathetic activity promotes glucose uptake for glycogen storage [152, 208]) have been shown to be controlled by the SCN in a different manner according to the time of the day [73, 209, 256]. Therefore, we propose that the SCN coordinates the organ functionality according to the active/resting period by an appropriate balance of the ANS input to the peripheral tissues according to the body compartment [71, 155, 236]. Interestingly, our group also demonstrated a communication between the SCN and the arcuate nucleus (ARC), the hypothalamic area that sense metabolic signals released into the circulation by the peripheral organs. Moreover vice versa, food-related signals detected by the ARC are also able to modulate the activity of the SCN [39]. All these observations taken together provide a picture of our clock continuously in need of being informed about the energy status of the body. Consequently it leads us to propose that in case of a "desynchronization" between external and internal signals (such as is the case in a nocturnal lifestyle which involves staying up late, night-eating behaviour, and skipping breakfast) this desynchronization will induce a disturbance of SCN activity [336]. This disturbed SCN activity will result in a disturbed SCN output which will eventually disturb the balance of the ANS [236]. Besides the contradictory signals of the L/D cycle *vs* feeding pattern, the autonomic outflow might also be disturbed as a result of an abnormal feedback from the periphery to the brain. For instance, in case of obesity [337] in which the metabolic balance is disturbed, a high caloric intake is combined with a low energy expenditure. Consequently, future research must be aimed at studying the relationship between the

structures that sense the condition of the periphery, such as arcuate and NTS, and the structures that are essential for generating the balance in the autonomic output of the brain, such as the SCN and PVN. In addition, human post-mortem research is essential to indicate which changes are present, in which structures, in order to get an understanding of the driving forces behind the development of diabetes type 2 and the metabolic syndrome.

References

1. Maung, S. L. & Follett, B. K. The endocrine control by luteinizing hormone of testosterone secretion from the testis of the Japanese quail. Gen Comp Endocrinol 36, 79-89 (1978).
2. Bronson, F. H. Mammalian reproduction: an ecological perspective. Biol Reprod 32, 1-26 (1985).
3. Hamilton, G. D. & Bronson, F. H. Food restriction and reproductive development in wild house mice. Biol Reprod 32, 773-8 (1985).
4. Wade, G. N. & Schneider, J. E. Metabolic fuels and reproduction in female mammals. Neurosci Biobehav Rev 16, 235-72 (1992).
5. Bolli, G. B. et al. Demonstration of a dawn phenomenon in normal human volunteers. Diabetes 33, 1150-3 (1984).
6. Stephan, F. K. & Zucker, I. Circadian rhythms in drinking behavior and locomotor activity of rats are eliminated by hypothalamic lesions. Proc Natl Acad Sci U S A 69, 1583-6 (1972).
7. Moore, R. Y. & Eichler, V. B. Loss of a circadian adrenal corticosterone rhythm following suprachiasmatic lesions in the rat. Brain Res 42, 201-6 (1972).
8. Moore, R. Y. & Klein, D. C. Visual pathways and the central neural control of a circadian rhythm in pineal serotonin N-acetyltransferase activity. Brain Res 71, 17-33 (1974).
9. Ibuka, N. & Kawamura, H. Loss of circadian rhythm in sleep-wakefulness cycle in the rat by suprachiasmatic nucleus lesions. Brain Res 96, 76-81 (1975).
10. Nagai, K., Nishio, T., Nakagawa, H., Nakamura, S. & Fukuda, Y. Effect of bilateral lesions of the suprachiasmatic nuclei on the circadian rhythm of food-intake. Brain Res 142, 384-9 (1978).
11. Inouye, S. T. & Kawamura, H. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. Proc Natl Acad Sci U S A 76, 5962-6 (1979).
12. Bos, N. P. & Mirmiran, M. Circadian rhythms in spontaneous neuronal discharges of the cultured suprachiasmatic nucleus. Brain Res 511, 158-62 (1990).
13. Welsh, D. K., Logothetis, D. E., Meister, M. & Reppert, S. M. Individual neurons dissociated from rat suprachiasmatic nucleus express independently phased circadian firing rhythms. Neuron 14, 697-706 (1995).
14. Schwartz, W. J. & Gainer, H. Suprachiasmatic nucleus: use of 14C-labeled deoxyglucose uptake as a functional marker. Science 197, 1089-91 (1977).
15. Schwartz, W. J., Lydic, R. & Moore-Ede, M. C. In vivo metabolic activity of the suprachiasmatic nuclei: non-uniform intranuclear distribution of 14C-labeled deoxyglucose uptake. Brain Res 424, 249-57 (1987).
16. Ralph, M. R., Foster, R. G., Davis, F. C. & Menaker, M. Transplanted suprachiasmatic nucleus determines circadian period. Science 247, 975-8 (1990).
17. Silver, R., LeSauter, J., Tresco, P. A. & Lehman, M. N. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. Nature 382, 810-3 (1996).
18. Meyer-Bernstein, E. L. et al. Effects of suprachiasmatic transplants on circadian rhythms of neuroendocrine function in golden hamsters. Endocrinology 140, 207-18 (1999).
19. Takahashi, J. S., Pinto, L. H. & Vitaterna, M. H. Forward and reverse genetic approaches to behavior in the mouse. Science 264, 1724-33 (1994).
20. Vitaterna, M. H. et al. Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264, 719-25 (1994).
21. Debruyne, J. P. et al. A Clock Shock: Mouse CLOCK Is Not Required for Circadian Oscillator Function. Neuron 50, 465-77 (2006).

22. van der Horst, G. T. et al. Mammalian Cry1 and Cry2 are essential for maintenance of circadian rhythms. Nature 398, 627-30 (1999).
23. Kume, K. et al. mCRY1 and mCRY2 are essential components of the negative limb of the circadian clock feedback loop. Cell 98, 193-205 (1999).
24. Shearman, L. P. et al. Interacting molecular loops in the mammalian circadian clock. Science 288, 1013-9 (2000).
25. Reppert, S. M. & Weaver, D. R. Molecular analysis of mammalian circadian rhythms. Annu Rev Physiol 63, 647-76 (2001).
26. Vielhaber, E., Eide, E., Rivers, A., Gao, Z. H. & Virshup, D. M. Nuclear entry of the circadian regulator mPER1 is controlled by mammalian casein kinase I epsilon. Mol Cell Biol 20, 4888-99 (2000).
27. Lee, C., Weaver, D. R. & Reppert, S. M. Direct association between mouse PERIOD and CK-Iepsilon is critical for a functioning circadian clock. Mol Cell Biol 24, 584-94 (2004).
28. Ueda, H. R. et al. A transcription factor response element for gene expression during circadian night. Nature 418, 534-9 (2002).
29. Preitner, N. et al. The orphan nuclear receptor REV-ERBalpha controls circadian transcription within the positive limb of the mammalian circadian oscillator. Cell 110, 251-60 (2002).
30. Onishi, H. et al. Rev-erbalpha gene expression in the mouse brain with special emphasis on its circadian profiles in the suprachiasmatic nucleus. J Neurosci Res 68, 551-7. (2002).
31. Akashi, M. & Takumi, T. The orphan nuclear receptor RORalpha regulates circadian transcription of the mammalian core-clock Bmal1. Nat Struct Mol Biol (2005).
32. Honma, S. et al. Dec1 and Dec2 are regulators of the mammalian molecular clock. Nature 419, 841-4. (2002).
33. Sato, F. et al. Functional analysis of the basic helix-loop-helix transcription factor DEC1 in circadian regulation. Eur J Biochem 271, 4409-19 (2004).
34. Jin, X. et al. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96, 57-68 (1999).
35. Inouye, S. T. Circadian rhythms of neuropeptides in the suprachiasmatic nucleus. Prog Brain Res 111, 75-90 (1996).
36. Card, J. P. & Moore, R. Y. Organization of lateral geniculate-hypothalamic connections in the rat. J Comp Neurol 284, 135-47 (1989).
37. Moore, R. Y. Entrainment pathways and the functional organization of the circadian system. Prog Brain Res 111, 103-19 (1996).
38. Card, J. P. & Moore, R. Y. Neuropeptide Y localization in the rat suprachiasmatic nucleus and periventricular hypothalamus. Neurosci Lett 88, 241-6 (1988).
39. Yi, C. X. et al. Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. Endocrinology 147, 283-94 (2006).
40. Tanaka, M. et al. Somatostatin neurons form a distinct peptidergic neuronal group in the rat suprachiasmatic nucleus: a double labeling in situ hybridization study. Neurosci Lett 215, 119-22 (1996).
41. Moore, R. Y., Speh, J. C. & Leak, R. K. Suprachiasmatic nucleus organization. Cell Tissue Res 309, 89-98 (2002).
42. Watanabe, K., Vanecek, J. & Yamaoka, S. In vitro entrainment of the circadian rhythm of vasopressin-releasing cells in suprachiasmatic nucleus by vasoactive intestinal polypeptide. Brain Res 877, 361-6 (2000).
43. Aida, R. et al. Gastrin-releasing peptide mediates photic entrainable signals to dorsal subsets of suprachiasmatic nucleus via induction of Period gene in mice. Mol Pharmacol 61, 26-34 (2002).
44. Liou, S. Y., Shibata, S., Iwasaki, K. & Ueki, S. Optic nerve stimulation-induced increase of release of 3H-glutamate and 3H-aspartate but not 3H-GABA from the suprachiasmatic nucleus in slices of rat hypothalamus. Brain Res Bull 16, 527-31 (1986).

45. de Vries, M. J., Nunes Cardozo, B., van der Want, J., de Wolf, A. & Meijer, J. H. Glutamate immunoreactivity in terminals of the retinohypothalamic tract of the brown Norwegian rat. Brain Res 612, 231-7 (1993).
46. Colwell, C. S., Foster, R. G. & Menaker, M. NMDA receptor antagonists block the effects of light on circadian behavior in the mouse. Brain Res 554, 105-10 (1991).
47. Abe, H., Rusak, B. & Robertson, H. A. NMDA and non-NMDA receptor antagonists inhibit photic induction of Fos protein in the hamster suprachiasmatic nucleus. Brain Res Bull 28, 831-5 (1992).
48. Earnest, D. J., Iadarola, M., Yeh, H. H. & Olschowka, J. A. Photic regulation of c-fos expression in neural components governing the entrainment of circadian rhythms. Exp Neurol 109, 353-61 (1990).
49. Best, J. D., Maywood, E. S., Smith, K. L. & Hastings, M. H. Rapid resetting of the mammalian circadian clock. J Neurosci 19, 828-35 (1999).
50. Castel, M., Belenky, M., Cohen, S., Wagner, S. & Schwartz, W. J. Light-induced c-Fos expression in the mouse suprachiasmatic nucleus: immunoelectron microscopy reveals co-localization in multiple cell types. Eur J Neurosci 9, 1950-60 (1997).
51. Buijs, R. M. et al. Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. Eur J Neurosci 11, 1535-44 (1999).
52. Scheer, F. A., Ter Horst, G. J., van Der Vliet, J. & Buijs, R. M. Physiological and anatomic evidence for regulation of the heart by suprachiasmatic nucleus in rats. Am J Physiol Heart Circ Physiol 280, H1391-9 (2001).
53. Shigeyoshi, Y. et al. Light-induced resetting of a mammalian circadian clock is associated with rapid induction of the mPer1 transcript. Cell 91, 1043-53 (1997).
54. Yan, L., Takekida, S., Shigeyoshi, Y. & Okamura, H. Per1 and Per2 gene expression in the rat suprachiasmatic nucleus: circadian profile and the compartment-specific response to light. Neuroscience 94, 141-50 (1999).
55. Kuhlman, S. J., Silver, R., Le Sauter, J., Bult-Ito, A. & McMahon, D. G. Phase resetting light pulses induce Per1 and persistent spike activity in a subpopulation of biological clock neurons. J Neurosci 23, 1441-50. (2003).
56. Yan, L. & Silver, R. Differential induction and localization of mPer1 and mPer2 during advancing and delaying phase shifts. Eur J Neurosci 16, 1531-40 (2002).
57. Dardente, H., Poirel, V. J., Klosen, P., Pevet, P. & Masson-Pevet, M. Per and neuropeptide expression in the rat suprachiasmatic nuclei: compartmentalization and differential cellular induction by light. Brain Res 958, 261-71 (2002).
58. Alves, S. E., Lopez, V., McEwen, B. S. & Weiland, N. G. Differential colocalization of estrogen receptor beta (ERbeta) with oxytocin and vasopressin in the paraventricular and supraoptic nuclei of the female rat brain: an immunocytochemical study. Proc Natl Acad Sci U S A 95, 3281-6 (1998).
59. Kalamatianos, T., Kallo, I., Piggins, H. D. & Coen, C. W. Expression of VIP and/or PACAP receptor mRNA in peptide synthesizing cells within the suprachiasmatic nucleus of the rat and in its efferent target sites. J Comp Neurol 475, 19-35 (2004).
60. Romijn, H. J., van Uum, J. F., Emmering, J., Goncharuk, V. & Buijs, R. M. Colocalization of VIP with AVP in neurons of the human paraventricular, supraoptic and suprachiasmatic nucleus. Brain Res 832, 47-53 (1999).
61. Buijs, R. M., Markman, M., Nunes-Cardoso, B., Hou, Y. X. & Shinn, S. Projections of the suprachiasmatic nucleus to stress-related areas in the rat hypothalamus: a light and electron microscopic study. J Comp Neurol 335, 42-54 (1993).
62. Perreau-Lenz, S. et al. Suprachiasmatic control of melatonin synthesis in rats: inhibitory and stimulatory mechanisms. Eur J Neurosci 17, 221-8 (2003).

63. Perreau-Lenz, S., Pevet, P., Buijs, R. M. & Kalsbeek, A. The biological clock: the bodyguard of temporal homeostasis. Chronobiol Int 21, 1-25 (2004).
64. Buijs, R. M. The anatomical basis for the expression of circadian rhythms: the efferent projections of the suprachiasmatic nucleus. Prog Brain Res 111, 229-40 (1996).
65. Buijs, R. M. & Kalsbeek, A. Hypothalamic integration of central and peripheral clocks. Nat Rev Neurosci 2, 521-6. (2001).
66. Kennaway, D. J. The role of circadian rhythmicity in reproduction. Hum Reprod Update 11, 91-101 (2005).
67. Kriegsfeld, L. J. & Silver, R. The regulation of neuroendocrine function: Timing is everything. Horm Behav (2006).
68. Saper, C. B., Loewy, A. D., Swanson, L. W. & Cowan, W. M. Direct hypothalamo-autonomic connections. Brain Res 117, 305-12 (1976).
69. Swanson, L. W. & Kuypers, H. G. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. J Comp Neurol 194, 555-70 (1980).
70. Bartness, T. J., Song, C. K. & Demas, G. E. SCN efferents to peripheral tissues: implications for biological rhythms. J Biol Rhythms 16, 196-204. (2001).
71. Buijs, R. M., Van Eden, C. G., Goncharuk, V. D. & Kalsbeek, A. The biological clock tunes the organs of the body: timing by hormones and the autonomic nervous system. J Endocrinol 177, 17-26. (2003).
72. Hermes, M. L., Coderre, E. M., Buijs, R. M. & Renaud, L. P. GABA and glutamate mediate rapid neurotransmission from suprachiasmatic nucleus to hypothalamic paraventricular nucleus in rat. J Physiol 496 (Pt 3), 749-57 (1996).
73. Kalsbeek, A., La Fleur, S., Van Heijningen, C. & Buijs, R. M. Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. J Neurosci 24, 7604-13 (2004).
74. Buijs, R. M., Chun, S. J., Niijima, A., Romijn, H. J. & Nagai, K. Parasympathetic and sympathetic control of the pancreas: a role for the suprachiasmatic nucleus and other hypothalamic centers that are involved in the regulation of food intake. J Comp Neurol 431, 405-23. (2001).
75. Kreier, F. et al. Selective parasympathetic innervation of subcutaneous and intra- abdominal fat - functional implications. J Clin Invest 110, 1243-50. (2002).
76. Kreier, F. et al. Tracing from fat tissue, liver, and pancreas: a neuroanatomical framework for the role of the brain in type 2 diabetes. Endocrinology 147, 1140-7 (2006).
77. Roland, B. L. & Sawchenko, P. E. Local origins of some GABAergic projections to the paraventricular and supraoptic nuclei of the hypothalamus in the rat. J Comp Neurol 332, 123-43 (1993).
78. Boudaba, C., Szabo, K. & Tasker, J. G. Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. J Neurosci 16, 7151-60 (1996).
79. Nagai, K. et al. SCN output drives the autonomic nervous system: with special reference to the autonomic function related to the regulation of glucose metabolism. Prog Brain Res 111, 253-72 (1996).
80. Nagai, K., Nagai, N., Sugahara, K., Niijima, A. & Nakagawa, H. Circadian rhythms and energy metabolism with special reference to the suprachiasmatic nucleus. Neurosci Biobehav Rev 18, 579-84 (1994).
81. Kalsbeek, A. et al. Melatonin sees the light: blocking GABA-ergic transmission in the paraventricular nucleus induces daytime secretion of melatonin. Eur J Neurosci 12, 3146-54 (2000).
82. Ishida, A. et al. Light activates the adrenal gland: timing of gene expression and glucocorticoid release. Cell Metab 2, 297-307 (2005).

83. Hastings, M. H. et al. Entrainment of the circadian clock. Prog Brain Res 111, 147-74 (1996).
84. Hastings, M. H., Field, M. D., Maywood, E. S., Weaver, D. R. & Reppert, S. M. Differential regulation of mPER1 and mTIM proteins in the mouse suprachiasmatic nuclei: new insights into a core clock mechanism. J Neurosci 19, RC11. (1999).
85. Rusak, B. & Groos, G. Suprachiasmatic stimulation phase shifts rodent circadian rhythms. Science 215, 1407-9 (1982).
86. Akiyama, M. et al. Inhibition of light- or glutamate-induced mPer1 expression represses the phase shifts into the mouse circadian locomotor and suprachiasmatic firing rhythms. J Neurosci 19, 1115-21 (1999).
87. Albrecht, U., Zheng, B., Larkin, D., Sun, Z. S. & Lee, C. C. MPer1 and mper2 are essential for normal resetting of the circadian clock. J Biol Rhythms 16, 100-4 (2001).
88. Tournier, B. B. et al. Photoperiod differentially regulates clock genes' expression in the suprachiasmatic nucleus of Syrian hamster. Neuroscience 118, 317-22 (2003).
89. la Fleur, S. E., Kalsbeek, A., Wortel, J., Fekkes, M. L. & Buijs, R. M. A daily rhythm in glucose tolerance: a role for the suprachiasmatic nucleus. Diabetes 50, 1237-43. (2001).
90. Pauly, J. E. & Scheving, L. E. Circadian rhythms in blood glucose and the effect of different lighting schedules, hypophysectomy, adrenal medullectomy and starvation. Am J Anat 120, 627-36 (1967).
91. Ahlersova, E., Ahlers, I., Smajda, B., Ivancinova, O. & Paulikova, E. Circadian rhythm of blood glucose and tissue glycogen in fed and fasted rats. Physiol Bohemoslov 29, 515-23 (1980).
92. La Fleur, S. E., Kalsbeek, A., Wortel, J. & Buijs, R. M. A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. J Neuroendocrinol 11, 643-52 (1999).
93. Carroll, K. F. & Nestel, P. J. Diurnal variation in glucose tolerance and in insulin secretion in man. Diabetes 22, 333-48 (1973).
94. Bellinger, L. L., Mendel VE & GP, a. M. Circadian insulin, GH, prolactin, corticosterone and glucose rhythms in fed and fasted rats. Horm Metab Res 7, 132-135 (1974).
95. Jolin, T. & Montes, A. Daily rhythm of plasma glucose and insulin levels in rats. Horm Res 4, 153-6 (1973).
96. Peret, J., Macaire, I. & Chanez, M. Schedule of protein ingestion, nitrogen and energy utilization and circadian rhythm of hepatic glycogen, plasma corticosterone and insulin in rats. J Nutr 103, 866-74 (1973).
97. Van Cauter, E. Diurnal and ultradian rhythms in human endocrine function: a minireview. Horm Res 34, 45-53 (1990).
98. Saito, M., Murakami, E. & Sudo, M. Circadian rhythm of disgestion and absorption in the small intestine of the rat and its relation to food intake. University Park Press, 565-573 (1977).
99. Murakami, E., Saito, M. & Suda, M. Contribution of diffusive pathway in intestinal absorption of glucose in rat under normal feeding condition. Experientia 33, 1469-70 (1977).
100. Ruiter, M. et al. The daily rhythm in plasma glucagon concentrations in the rat is modulated by the biological clock and by feeding behavior. Diabetes 52, 1709-15 (2003).
101. Kalsbeek, A. & Strubbe, J. H. Circadian control of insulin secretion is independent of the temporal distribution of feeding. Physiol Behav 63, 553-8 (1998).
102. Jarrett, R. J., Baker, I. A., Keen, H. & Oakley, N. W. Diurnal variation in oral glucose tolerance: blood sugar and plasma insulin levels morning, afternoon, and evening. Br Med J 1, 199-201 (1972).
103. Whichelow, M. J. et al. Diurnal variation in response to intravenous glucose. Br Med J 1, 488-91 (1974).
104. Lee, A., Ader, M., Bray, G. A. & Bergman, R. N. Diurnal variation in glucose tolerance. Cyclic suppression of insulin action and insulin secretion in normal-weight, but not obese, subjects. Diabetes 41, 742-9 (1992).

105. Yamamoto, H., Nagai, K. & Nakagawa, H. Bilateral lesions of the suprachiasmatic nucleus enhance glucose tolerance in rats. Biomed. Res. 5, 47-54 (1984).
106. Morgan, L. M., Aspostolakou, F., Wright, J. & Gama, R. Diurnal variations in peripheral insulin resistance and plasma non-esterified fatty acid concentrations: a possible link? Ann Clin Biochem 36 (Pt 4), 447-50 (1999).
107. Feneberg, R. & Lemmer, B. Circadian rhythm of glucose uptake in cultures of skeletal muscle cells and adipocytes in Wistar-Kyoto, Wistar, Goto-Kakizaki, and spontaneously hypertensive rats. Chronobiol Int 21, 521-38 (2004).
108. Corssmit, E. P., Romijn, J. A. & Sauerwein, H. P. Review article: Regulation of glucose production with special attention to nonclassical regulatory mechanisms: a review. Metabolism 50, 742-55 (2001).
109. Cherrington, A. D., Chiasson, J. L., Liljenquist, J. E., Lacy, W. W. & Park, C. R. Control of hepatic glucose output by glucagon and insulin in the intact dog. Biochem Soc Symp, 31-45 (1978).
110. Cherrington, A. D., Williams, P. E., Shulman, G. I. & Lacy, W. W. Differential time course of glucagon's effect on glycogenolysis and gluconeogenesis in the conscious dog. Diabetes 30, 180-7 (1981).
111. Beale, E., Andreone, T., Koch, S., Granner, M. & Granner, D. Insulin and glucagon regulate cytosolic phosphoenolpyruvate carboxykinase (GTP) mRNA in rat liver. Diabetes 33, 328-32 (1984).
112. Pilkis, S. J. & Granner, D. K. Molecular physiology of the regulation of hepatic gluconeogenesis and glycolysis. Annu Rev Physiol 54, 885-909 (1992).
113. Yamamoto, H., Nagai, K. & Nakagawa, H. Role of SCN in daily rhythms of plasma glucose, FFA, insulin and glucagon. Chronobiol Int 4, 483-91 (1987).
114. Chu, C. A., Sindelar, D. K., Neal, D. W. & Cherrington, A. D. Direct effects of catecholamines on hepatic glucose production in conscious dog are due to glycogenolysis. Am J Physiol 271, E127-37 (1996).
115. Chu, C. A. et al. Comparison of the direct and indirect effects of epinephrine on hepatic glucose production. J Clin Invest 99, 1044-56 (1997).
116. Berneis, K. & Keller, U. Metabolic actions of growth hormone: direct and indirect. Baillieres Clin Endocrinol Metab 10, 337-52 (1996).
117. Tayek, J. A., Bulcavage, L. & Chlebowski, R. T. Relationship of hepatic glucose production to growth hormone and severity of malnutrition in a population with colorectal carcinoma. Cancer Res 50, 2119-22 (1990).
118. Sistare, F. D. & Haynes, R. C., Jr. Acute stimulation by glucocorticoids of gluconeogenesis from lactate/pyruvate in isolated hepatocytes from normal and adrenalectomized rats. J Biol Chem 260, 12754-60 (1985).
119. Exton, J. H. Regulation of gluconeogenesis by glucocorticoids. Monogr Endocrinol 12, 535-46 (1979).
120. Pincus, S. M. et al. Females secrete growth hormone with more process irregularity than males in both humans and rats. Am J Physiol 270, E107-15 (1996).
121. Clark, R. G., Chambers, G., Lewin, J. & Robinson, I. C. Automated repetitive microsampling of blood: growth hormone profiles in conscious male rats. J Endocrinol 111, 27-35 (1986).
122. De Boer, S. F. & Van der Gugten, J. Daily variations in plasma noradrenaline, adrenaline and corticosterone concentrations in rats. Physiol Behav 40, 323-8 (1987).
123. Shamoon, H., Hendler, R. & Sherwin, R. S. Altered responsiveness to cortisol, epinephrine, and glucagon in insulin-infused juvenile-onset diabetics. A mechanism for diabetic instability. Diabetes 29, 284-91 (1980).
124. Bright, G. M., Melton, T. W., Rogol, A. D. & Clarke, W. L. Failure of cortisol blockade to inhibit early morning increases in basal insulin requirements in fasting insulin-dependent diabetics. Diabetes 29, 662-4 (1980).

125. Woerle, H. J. et al. Mechanisms for abnormal postprandial glucose metabolism in type 2 diabetes. Am J Physiol Endocrinol Metab 290, E67-E77 (2006).
126. Roden, M. et al. Effects of free fatty acid elevation on postabsorptive endogenous glucose production and gluconeogenesis in humans. Diabetes 49, 701-7 (2000).
127. Randle, P. J. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. Diabetes Metab Rev 14, 263-83 (1998).
128. Chen, X., Iqbal, N. & Boden, G. The effects of free fatty acids on gluconeogenesis and glycogenolysis in normal subjects. J Clin Invest 103, 365-72 (1999).
129. Morgan, K., Obici, S. & Rossetti, L. Hypothalamic responses to long-chain fatty acids are nutritionally regulated. J Biol Chem 279, 31139-48 (2004).
130. Dallman, M. F. et al. Starvation: early signals, sensors, and sequelae. Endocrinology 140, 4015-23. (1999).
131. Cornish, S. & Cawthorne, M. A. Fatty acid synthesis in mice during the 24hr cycle and during meal-feeding. Horm Metab Res 10, 286-90 (1978).
132. Hems, D. A., Rath, E. A. & Verrinder, T. R. Fatty acid synthesis in liver and adipose tissue of normal and genetically obese (ob/ob) mice during the 24-hour cycle. Biochem J 150, 167-73 (1975).
133. Zardoya, R. et al. Lipogenic activities in rat liver are subjected to circadian rhythms. Rev Esp Fisiol 50, 239-44 (1994).
134. Benavides, A., Siches, M. & Llobera, M. Circadian rhythms of lipoprotein lipase and hepatic lipase activities in intermediate metabolism of adult rat. Am J Physiol 275, R811-7 (1998).
135. Tsutsumi, K., Inoue, Y. & Kondo, Y. The relationship between lipoprotein lipase activity and respiratory quotient of rats in circadian rhythms. Biol Pharm Bull 25, 1360-3 (2002).
136. Lemberger, T. et al. Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm. J Biol Chem 271, 1764-9 (1996).
137. Martin, R. J., Stolz, D. J. & Buck, D. C. Diurnal changes in adipose and liver tissue metabolism of lean and obese Zucker rats. J Nutr 109, 412-7 (1979).
138. Guerre-Millo, M. Extending the glucose/fatty acid cycle: a glucose/adipose tissue cycle. Biochem Soc Trans 31, 1161-4 (2003).
139. Kalsbeek, A. et al. The suprachiasmatic nucleus generates the diurnal changes in plasma leptin levels. Endocrinology 142, 2677-85. (2001).
140. Luheshi, G. N., Gardner, J. D., Rushforth, D. A., Loudon, A. S. & Rothwell, N. J. Leptin actions on food intake and body temperature are mediated by IL-1. Proc Natl Acad Sci U S A 96, 7047-52. (1999).
141. Pocai, A. et al. Central leptin acutely reverses diet-induced hepatic insulin resistance. Diabetes 54, 3182-9 (2005).
142. Shimomura, I., Hammer, R. E., Ikemoto, S., Brown, M. S. & Goldstein, J. L. Leptin reverses insulin resistance and diabetes mellitus in mice with congenital lipodystrophy. Nature 401, 73-6 (1999).
143. Liu, L. et al. Intracerebroventricular leptin regulates hepatic but not peripheral glucose fluxes. J Biol Chem 273, 31160-7 (1998).
144. Combs, T. P., Berg, A. H., Obici, S., Scherer, P. E. & Rossetti, L. Endogenous glucose production is inhibited by the adipose-derived protein Acrp30. J Clin Invest 108, 1875-81 (2001).
145. Havel, P. J., Townsend, R., Chaump, L. & Teff, K. High-fat meals reduce 24-h circulating leptin concentrations in women. Diabetes 48, 334-41 (1999).
146. Chacon, F. et al. 24-hour changes in ACTH, corticosterone, growth hormone, and leptin levels in young male rats subjected to calorie restriction. Chronobiol Int 22, 253-65 (2005).
147. Ahren, B. Diurnal variation in circulating leptin is dependent on gender, food intake and circulating insulin in mice. Acta Physiol Scand 169, 325-31 (2000).

148. Gavrila, A. et al. Diurnal and ultradian dynamics of serum adiponectin in healthy men: comparison with leptin, circulating soluble leptin receptor, and cortisol patterns. J Clin Endocrinol Metab 88, 2838-43 (2003).
149. Yamaguchi, N. Sympathoadrenal system in neuroendocrine control of glucose: mechanisms involved in the liver, pancreas, and adrenal gland under hemorrhagic and hypoglycemic stress. Can J Physiol Pharmacol 70, 167-206 (1992).
150. Frohman, L. A. & Bernardis, L. L. Effect of hypothalamic stimulation on plasma glucose, insulin, and glucagon levels. Am J Physiol 221, 1596-603 (1971).
151. Shimazu, T. & Ogasawara, S. Effects of hypothalamic stimulation on gluconeogenesis and glycolysis in rat liver. Am J Physiol 228, 1787-93 (1975).
152. Shimazu, T. & Fujimoto, T. Regulation of glycogen metabolism in liver by the autonomic nervous system. IV. Neural control of glycogen biosynthesis. Biochim Biophys Acta 252, 18-27 (1971).
153. Shimazu, T. Glycogen synthetase activity in liver: regulation by the autonomic nerves. Science 156, 1256-7 (1967).
154. la Fleur, S. E., Kalsbeek, A., Wortel, J. & Buijs, R. M. Polysynaptic neural pathways between the hypothalamus, including the suprachiasmatic nucleus, and the liver. Brain Res 871, 50-6. (2000).
155. Buijs, R. M. et al. The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. J Comp Neurol 464, 36-48 (2003).
156. Metz, R. P., Qu, X., Laffin, B., Earnest, D. & Porter, W. W. Circadian clock and cell cycle gene expression in mouse mammary epithelial cells and in the developing mouse mammary gland. Dev Dyn 235, 263-71 (2006).
157. Fu, L., Patel, M. S., Bradley, A., Wagner, E. F. & Karsenty, G. The molecular clock mediates leptin-regulated bone formation. Cell 122, 803-15 (2005).
158. Teboul, M. et al. Atypical patterns of circadian clock gene expression in human peripheral blood mononuclear cells. J Mol Med 83, 693-9 (2005).
159. Simonneaux, V. et al. Daily rhythm and regulation of clock gene expression in the rat pineal gland. Brain Res Mol Brain Res 120, 164-72 (2004).
160. Bittman, E. L., Doherty, L., Huang, L. & Paroskie, A. Period gene expression in mouse endocrine tissues. Am J Physiol Regul Integr Comp Physiol 285, R561-9 (2003).
161. Storch, K. F. et al. Extensive and divergent circadian gene expression in liver and heart. Nature 417, 78-83. (2002).
162. Sakamoto, K. et al. Multitissue circadian expression of rat period homolog (rPer2) mRNA is governed by the mammalian circadian clock, the suprachiasmatic nucleus in the brain. J Biol Chem 273, 27039-42. (1998).
163. Alvarez, J. D. & Sehgal, A. The thymus is similar to the testis in its pattern of circadian clock gene expression. J Biol Rhythms 20, 111-21 (2005).
164. Muhlbauer, E., Wolgast, S., Finckh, U., Peschke, D. & Peschke, E. Indication of circadian oscillations in the rat pancreas. FEBS Lett 564, 91-6 (2004).
165. Zvonic, S. et al. Characterization of peripheral circadian clocks in adipose tissues. Diabetes 55, 962-70 (2006).
166. Panda, S. et al. Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307-20. (2002).
167. Reppert, S. M. & Weaver, D. R. Coordination of circadian timing in mammals. Nature 418, 935-41. (2002).
168. Damiola, F. et al. Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. Genes Dev 14, 2950-61. (2000).

169. Marchant, E. G. & Mistlberger, R. E. Anticipation and entrainment to feeding time in intact and SCN-ablated C57BL/6j mice. Brain Res 765, 273-82 (1997).
170. Hara, R. et al. Restricted feeding entrains liver clock without participation of the suprachiasmatic nucleus. Genes Cells 6, 269-78. (2001).
171. Stokkan, K. A., Yamazaki, S., Tei, H., Sakaki, Y. & Menaker, M. Entrainment of the circadian clock in the liver by feeding. Science 291, 490-3. (2001).
172. Kobayashi, H., Oishi, K., Hanai, S. & Ishida, N. Effect of feeding on peripheral circadian rhythms and behaviour in mammals. Genes Cells 9, 857-64 (2004).
173. Schibler, U., Ripperger, J. & Brown, S. A. Peripheral circadian oscillators in mammals: time and food. J Biol Rhythms 18, 250-60 (2003).
174. Kuriyama, K., Sasahara, K., Kudo, T. & Shibata, S. Daily injection of insulin attenuated impairment of liver circadian clock oscillation in the streptozotocin-treated diabetic mouse. FEBS Lett 572, 206-10 (2004).
175. Hirota, T. et al. Glucose down-regulates Per1 and Per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. J Biol Chem 277, 44244-51. (2002).
176. Grechez-Cassiau, A. et al. The transcriptional repressor STRA13 regulates a subset of peripheral circadian outputs. J Biol Chem 279, 1141-50 (2004).
177. Balsalobre, A., Marcacci, L. & Schibler, U. Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. Curr Biol 10, 1291-4. (2000).
178. Guo, H., Brewer, J. M., Champhekar, A., Harris, R. B. & Bittman, E. L. Differential control of peripheral circadian rhythms by suprachiasmatic-dependent neural signals. Proc Natl Acad Sci U S A 102, 3111-6 (2005).
179. Balsalobre, A. et al. Resetting of circadian time in peripheral tissues by glucocorticoid signaling. Science 289, 2344-7. (2000).
180. Terazono, H. et al. Adrenergic regulation of clock gene expression in mouse liver. Proc Natl Acad Sci U S A 100, 6795-800. (2003).
181. Le Minh, N., Damiola, F., Tronche, F., Schutz, G. & Schibler, U. Glucocorticoid hormones inhibit food-induced phase-shifting of peripheral circadian oscillators. Embo J 20, 7128-36. (2001).
182. Brown, S. A., Zumbrunn, G., Fleury-Olela, F., Preitner, N. & Schibler, U. Rhythms of mammalian body temperature can sustain peripheral circadian clocks. Curr Biol 12, 1574-83. (2002).
183. Schibler, U. & Naef, F. Cellular oscillators: rhythmic gene expression and metabolism. Curr Opin Cell Biol 17, 223-9 (2005).
184. Schibler, U. & Brown, S. A. Enlightening the adrenal gland. Cell Metab 2, 278-81 (2005).
185. Radziuk, J. & Pye, S. Hepatic glucose uptake, gluconeogenesis and the regulation of glycogen synthesis. Diabetes Metab Res Rev 17, 250-72 (2001).
186. Kida, K. et al. The circadian change of gluconeogenesis in the liver in vivo in fed rats. J Biochem (Tokyo) 88, 1009-13 (1980).
187. Phillips, L. J. & Berry, L. J. Circadian rhythm of mouse liver phosphoenolpyruvate carboxykinase. Am J Physiol 218, 1440-4 (1970).
188. Iitaka, C., Miyazaki, K., Akaike, T. & Ishida, N. A role for glycogen synthase kinase-3beta in the mammalian circadian clock. J Biol Chem 280, 29397-402 (2005).
189. Oishi, K. et al. Genome-wide Expression Analysis Reveals 100 Adrenal Gland-dependent Circadian Genes in the Mouse Liver. DNA Res 12, 191-202 (2005).
190. Canaple, L. et al. Reciprocal regulation of BMAL1 and PPAR{alpha} defines a novel positive feedback loop in the rodent liver circadian clock. Mol Endocrinol (2006).
191. Staels, B. When the Clock stops ticking, metabolic syndrome explodes. Nat Med 12, 54-5; discussion 55 (2006).
192. Kalsbeek, A. & Buijs, R. M. Output pathways of the mammalian suprachiasmatic nucleus: coding circadian time by transmitter selection and specific targeting. Cell Tissue Res 309, 109-18. (2002).

193. Albrecht, U. The mammalian circadian clock: a network of gene expression. Front Biosci 9, 48-55 (2004).
194. Okamura, H. et al. Photic induction of mPer1 and mPer2 in cry-deficient mice lacking a biological clock. Science 286, 2531-4. (1999).
195. Albus, H. et al. Cryptochrome-deficient mice lack circadian electrical activity in the suprachiasmatic nuclei. Curr Biol 12, 1130-3. (2002).
196. Glossop, N. R. & Hardin, P. E. Central and peripheral circadian oscillator mechanisms in flies and mammals. J Cell Sci 115, 3369-77. (2002).
197. Yamazaki, S. et al. Resetting central and peripheral circadian oscillators in transgenic rats. Science 288, 682-5. (2000).
198. Hirota, T. & Fukada, Y. Resetting mechanism of central and peripheral circadian clocks in mammals. Zoolog Sci 21, 359-68 (2004).
199. Davidson, A. J., Yamazaki, S. & Menaker, M. SCN: ringmaster of the circadian circus or conductor of the circadian orchestra? Novartis Found Symp 253, 110-21; discussion 121-5, 281-4 (2003).
200. Yoo, S. H. et al. A noncanonical E-box enhancer drives mouse Period2 circadian oscillations in vivo. Proc Natl Acad Sci U S A 102, 2608-13 (2005).
201. La Fleur, S. E. Daily rhythms in glucose metabolism: suprachiasmatic nucleus output to peripheral tissue. J Neuroendocrinol 15, 315-22 (2003).
202. Lang, C. H. Inhibition of central GABAA receptors enhances hepatic glucose production and peripheral glucose uptake. Brain Res Bull 37, 611-6 (1995).
203. Steffens, A. B. A method for frequent sampling blood and continuous infusion of fluids in the rat without disturbing the animal. Physiol Behav 4, 833-836 (1969).
204. Bardsley, W. G., Bukhary, N. A. J., Ferguson, M. W. J., Cachaza, J. A. & Burguillo, F. J. Evaluation of the model discrimination parameter estimation and goodness of fit in nonlinear regression problems by test statistics distributions. Computers Chem. 19, 75-84 (1995).
205. Zylka, M. J., Shearman, L. P., Weaver, D. R. & Reppert, S. M. Three period homologs in mammals: differential light responses in the suprachiasmatic circadian clock and oscillating transcripts outside of brain. Neuron 20, 1103-10. (1998).
206. Miki, H. et al. Total parenteral nutrition entrains the central and peripheral circadian clocks. Neuroreport 14, 1457-61 (2003).
207. Yamamoto, T. et al. Transcriptional oscillation of canonical clock genes in mouse peripheral tissues. BMC Mol Biol 5, 18 (2004).
208. Shimazu, T. Reciprocal innervation of the liver: its significance in metabolic control. Adv Metab Disord 10, 355-84 (1983).
209. Nagai, K., Fujii, T., Inoue, S., Takamura, Y. & Nakagawa, H. Electrical stimulation of the suprachiasmatic nucleus of the hypothalamus causes hyperglycemia. Horm Metab Res 20, 37-9 (1988).
210. Tappy, L., Jequier, E. & Schneiter, P. Autoregulation of Glucose Production. News Physiol Sci 15, 198-202 (2000).
211. Davidson, A. J., Stokkan, K. A., Yamazaki, S. & Menaker, M. Food-anticipatory activity and liver per1-luc activity in diabetic transgenic rats. Physiol Behav 76, 21-6. (2002).
212. Kudo, T. et al. Night-time restricted feeding normalises clock genes and Pai-1 gene expression in the db/db mouse liver. Diabetologia (2004).
213. Yoo, S. H. et al. PERIOD2:LUCIFERASE real-time reporting of circadian dynamics reveals persistent circadian oscillations in mouse peripheral tissues. Proc Natl Acad Sci U S A (2004).
214. Pocai, A. et al. Hypothalamic K(ATP) channels control hepatic glucose production. Nature 434, 1026-31 (2005).
215. Lam, T. K. et al. Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. Nat Med 11, 320-7 (2005).

216. Matsuo, T. et al. Control mechanism of the circadian clock for timing of cell division in vivo. Science 302, 255-9 (2003).
217. Cailotto, C. et al. The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? Eur J Neurosci 22, 2531-40 (2005).
218. Akhtar, R. A. et al. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 12, 540-50 (2002).
219. Oishi, K. et al. Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J Biol Chem 278, 41519-27 (2003).
220. Pocai, A., Obici, S., Schwartz, G. J. & Rossetti, L. A brain-liver circuit regulates glucose homeostasis. Cell Metab 1, 53-61 (2005).
221. la Fleur, S. E., Ji, H., Manalo, S. L., Friedman, M. I. & Dallman, M. F. The hepatic vagus mediates fat-induced inhibition of diabetic hyperphagia. Diabetes 52, 2321-30 (2003).
222. Colle, I., Van Vlierberghe, H., Troisi, R. & De Hemptinne, B. Transplanted liver: consequences of denervation for liver functions. Anat Rec A Discov Mol Cell Evol Biol 280, 924-31 (2004).
223. Passonneau, J. V. & Lauderdale, V. R. A comparison of three methods of glycogen measurement in tissues. Anal Biochem 60, 405-12 (1974).
224. Carr, R. S. & Neff, J. M. Quantitative semi-automated enzymatic assay for tissue glycogen. Comp Biochem Physiol B 77, 447-9 (1984).
225. Xue, C. et al. Isolated hepatic cholinergic denervation impairs glucose and glycogen metabolism. J Surg Res 90, 19-25 (2000).
226. Armstrong, S. A chronometric approach to the study of feeding behavior. Neurosci Biobehav Rev 4, 27-53 (1980).
227. Escobar, C., Diaz-Munoz, M., Encinas, F. & Aguilar-Roblero, R. Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. Am J Physiol 274, R1309-16 (1998).
228. Kita, Y. et al. Implications of circadian gene expression in kidney, liver and the effects of fasting on pharmacogenomic studies. Pharmacogenetics 12, 55-65. (2002).
229. Paulikova, E., Ahlers, I. & Praslicka, M. Circadian changes of gluconeogenic enzymes in irradiated rats. Physiol Bohemoslov 34, 351-7 (1985).
230. Gupta, G. S., Sodhi, J. & Gupta, V. P. Circadian rhythmometric analysis of hepatic phosphohydrolases with special reference to glucose-6-phosphatase and inorganic pyrophosphatase. Indian J Biochem Biophys 31, 206-10 (1994).
231. van Schaftingen, E. & Gerin, I. The glucose-6-phosphatase system. Biochem J 362, 513-32 (2002).
232. Pilkis, S. J., Weber, I. T., Harrison, R. W. & Bell, G. I. Glucokinase: structural analysis of a protein involved in susceptibility to diabetes. J Biol Chem 269, 21925-8 (1994).
233. Jeanrenaud, B. Insulin, corticosterone and the autonomic nervous system in animal obesities: a viewpoint. Diabetologia 38, 998-1002 (1995).
234. Jeanrenaud, B. Central nervous system and peripheral abnormalities: clues to the understanding of obesity and NIDDM. Diabetologia 37 Suppl 2, S170-8 (1994).
235. Frontoni, S. et al. Early autonomic dysfunction in glucose-tolerant but insulin-resistant offspring of type 2 diabetic patients. Hypertension 41, 1223-7 (2003).
236. Kreier, F. et al. Hypothesis: shifting the equilibrium from activity to food leads to autonomic unbalance and the metabolic syndrome. Diabetes 52, 2652-6 (2003).
237. Barzilai, N. & Rossetti, L. Role of glucokinase and glucose-6-phosphatase in the acute and chronic regulation of hepatic glucose fluxes by insulin. J Biol Chem 268, 25019-25 (1993).
238. Minassian, C., Tarpin, S. & Mithieux, G. Role of glucose-6 phosphatase, glucokinase, and glucose-6 phosphate in liver insulin resistance and its correction by metformin. Biochem Pharmacol 55, 1213-9 (1998).

239. Clore, J. N., Stillman, J. & Sugerman, H. Glucose-6-phosphatase flux in vitro is increased in type 2 diabetes. Diabetes 49, 969-74 (2000).
240. Perseghin, G. et al. Regulation of glucose homeostasis in humans with denervated livers. J Clin Invest 100, 931-41 (1997).
241. Schneiter, P. et al. Postprandial hepatic glycogen synthesis in liver transplant recipients. Transplantation 69, 978-81 (2000).
242. Ishikawa, K. & Shimazu, T. Daily rhythms of glycogen synthetase and phosphorylase activities in rat liver: influence of food and light. Life Sci 19, 1873-8 (1976).
243. Kaminsky, Y. G. & Kosenko, E. A. Diurnal rhythms in liver carbohydrate metabolism. Comparative aspects and critical review. Comp Biochem Physiol B 86, 763-84 (1987).
244. Davidson, A. J., Castanon-Cervantes, O. & Stephan, F. K. Daily oscillations in liver function: diurnal vs circadian rhythmicity. Liver Int 24, 179-86 (2004).
245. Drijfhout, W. J. et al. A telemetry study on the chronic effects of microdialysis probe implantation on the activity pattern and temperature rhythm of the rat. J Neurosci Methods 61, 191-6 (1995).
246. Kalsbeek, A., van Heerikhuize, J. J., Wortel, J. & Buijs, R. M. A diurnal rhythm of stimulatory input to the hypothalamo-pituitary-adrenal system as revealed by timed intrahypothalamic administration of the vasopressin V1 antagonist. J Neurosci 16, 5555-65 (1996).
247. Martin, D. S., Segura, T. & Haywood, J. R. Cardiovascular responses to bicuculline in the paraventricular nucleus of the rat. Hypertension 18, 48-55 (1991).
248. Granner, D. & Pilkis, S. The genes of hepatic glucose metabolism. J Biol Chem 265, 10173-6 (1990).
249. Munoz, M. E. & Ponce, E. Pyruvate kinase: current status of regulatory and functional properties. Comp Biochem Physiol B Biochem Mol Biol 135, 197-218 (2003).
250. Moore, M. C., Connolly, C. C. & Cherrington, A. D. Autoregulation of hepatic glucose production. Eur J Endocrinol 138, 240-8 (1998).
251. Jones, C. G., Hothi, S. K. & Titheradge, M. A. Effect of dexamethasone on gluconeogenesis, pyruvate kinase, pyruvate carboxylase and pyruvate dehydrogenase flux in isolated hepatocytes. Biochem J 289 (Pt 3), 821-8 (1993).
252. Gomis, R. R. et al. Glucose 6-phosphate produced by gluconeogenesis and by glucokinase is equally effective in activating hepatic glycogen synthase. J Biol Chem 278, 9740-6 (2003).
253. van Schaftingen, E., Vandercammen, A., Detheux, M. & Davies, D. R. The regulatory protein of liver glucokinase. Adv Enzyme Regul 32, 133-48 (1992).
254. Massillon, D. Regulation of the glucose-6-phosphatase gene by glucose occurs by transcriptional and post-transcriptional mechanisms. Differential effect of glucose and xylitol. J Biol Chem 276, 4055-62 (2001).
255. Reddy, A. B. et al. Circadian orchestration of the hepatic proteome. Curr Biol 16, 1107-15 (2006).
256. Shibata, S. Neural regulation of the hepatic circadian rhythm. Anat Rec A Discov Mol Cell Evol Biol 280, 901-9 (2004).
257. Moore, R. Y. Organization and function of a central nervous system circadian oscillator: the suprachiasmatic hypothalamic nucleus. Fed Proc 42, 2783-9 (1983).
258. Weaver, D. R. The suprachiasmatic nucleus: a 25-year retrospective. J Biol Rhythms 13, 100-12 (1998).
259. Green, D. J. & Gillette, R. Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. Brain Res 245, 198-200 (1982).
260. Groos, G. & Hendriks, J. Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro. Neurosci Lett 34, 283-8 (1982).
261. Buijs, R. M., Hermes, M. H. & Kalsbeek, A. The suprachiasmatic nucleus-paraventricular nucleus interactions: a bridge to the neuroendocrine and autonomic nervous system. Prog Brain Res 119, 365-82 (1998).

262. Larsen, P. J., Enquist, L. W. & Card, J. P. Characterization of the multisynaptic neuronal control of the rat pineal gland using viral transneuronal tracing. Eur J Neurosci 10, 128-45 (1998).
263. Teclemariam-Mesbah, R., Ter Horst, G. J., Postema, F., Wortel, J. & Buijs, R. M. Anatomical demonstration of the suprachiasmatic nucleus-pineal pathway. J Comp Neurol 406, 171-82 (1999).
264. Kalsbeek, A., Cutrera, R. A., Van Heerikhuize, J. J., Van Der Vliet, J. & Buijs, R. M. GABA release from suprachiasmatic nucleus terminals is necessary for the light-induced inhibition of nocturnal melatonin release in the rat. Neuroscience 91, 453-61 (1999).
265. Foulkes, N. S., Whitmore, D. & Sassone-Corsi, P. Rhythmic transcription: the molecular basis of circadian melatonin synthesis. Biol Cell 89, 487-94 (1997).
266. Korf, H. W. & von Gall, C. Mice, melatonin and the circadian system. Mol Cell Endocrinol 252, 57-68 (2006).
267. Slotten, H. A., Pitrosky, B. & Pevet, P. Entrainment of rat circadian rhythms by melatonin does not depend on the serotonergic afferents to the suprachiasmatic nuclei. Brain Res 876, 10-6 (2000).
268. Gerendai, I. & Halasz, B. Central nervous system structures connected with the endocrine glands. findings obtained with the viral transneuronal tracing technique. Exp Clin Endocrinol Diabetes 108, 389-95 (2000).
269. Niijima, A., Nagai, K., Nagai, N. & Akagawa, H. Effects of light stimulation on the activity of the autonomic nerves in anesthetized rats. Physiol Behav 54, 555-61 (1993).
270. Tessonneaud, A., Locatelli, A., Caldani, M. & Viguier-Martinez, M. C. Bilateral lesions of the suprachiasmatic nuclei alter the nocturnal melatonin secretion in sheep. J Neuroendocrinol 7, 145-52 (1995).
271. Paul, K. N. et al. Tetrodotoxin administration in the suprachiasmatic nucleus prevents NMDA-induced reductions in pineal melatonin without influencing Per1 and Per2 mRNA levels. Eur J Neurosci 19, 2808-14 (2004).
272. Takekida, S., Yan, L., Maywood, E. S., Hastings, M. H. & Okamura, H. Differential adrenergic regulation of the circadian expression of the clock genes Period1 and Period2 in the rat pineal gland. Eur J Neurosci 12, 4557-61 (2000).
273. Schioth, H. B. et al. Remarkable synteny conservation of melanocortin receptors in chicken, human, and other vertebrates. Genomics 81, 504-9 (2003).
274. Peri, A. et al. Variable expression of the transcription factors cAMP response element-binding protein and inducible cAMP early repressor in the normal adrenal cortex and in adrenocortical adenomas and carcinomas. J Clin Endocrinol Metab 86, 5443-9 (2001).
275. Baig, A. H. et al. Agonist activated adrenocorticotropin receptor internalizes via a clathrin-mediated G protein receptor kinase dependent mechanism. Endocr Res 28, 281-9 (2002).
276. Izumi, S., Abe, K., Hayashi, T., Nakane, P. K. & Koji, T. Immunohistochemical localization of the ACTH (MC-2) receptor in the rat placenta and adrenal gland. Arch Histol Cytol 67, 443-53 (2004).
277. Mountjoy, K. G., Robbins, L. S., Mortrud, M. T. & Cone, R. D. The cloning of a family of genes that encode the melanocortin receptors. Science 257, 1248-51 (1992).
278. Griffon, N. et al. Molecular cloning and characterization of the rat fifth melanocortin receptor. Biochem Biophys Res Commun 200, 1007-14 (1994).
279. Liakos, P., Chambaz, E. M., Feige, J. J. & Defaye, G. Expression of ACTH receptors (MC2-R and MC5-R) in the glomerulosa and the fasciculata-reticularis zones of bovine adrenal cortex. Endocr Res 24, 427-32 (1998).
280. Engeland, W. C. & Arnhold, M. M. Neural circuitry in the regulation of adrenal corticosterone rhythmicity. Endocrine 28, 325-32 (2005).
281. Arendt, J., Deacon, S., English, J., Hampton, S. & Morgan, L. Melatonin and adjustment to phase shift. J Sleep Res 4, 74-79 (1995).

282. Uno, K. et al. Neuronal pathway from the liver modulates energy expenditure and systemic insulin sensitivity. Science 312, 1656-9 (2006).
283. Dunlap, J. C., Loros, J. J., Liu, Y. & Crosthwaite, S. K. Eukaryotic circadian systems: cycles in common. Genes Cells 4, 1-10 (1999).
284. Young, M. W. & Kay, S. A. Time zones: a comparative genetics of circadian clocks. Nat Rev Genet 2, 702-15 (2001).
285. Lee, C. C. Tumor suppression by the mammalian Period genes. Cancer Causes Control 17, 525-30 (2006).
286. Nagoshi, E. et al. Circadian gene expression in individual fibroblasts; cell-autonomous and self-sustained oscillators pass time to daughter cells. Cell 119, 693-705 (2004).
287. Welsh, D. K., Yoo, S. H., Liu, A. C., Takahashi, J. S. & Kay, S. A. Bioluminescence imaging of individual fibroblasts reveals persistent, independently phased circadian rhythms of clock gene expression. Curr Biol 14, 2289-95 (2004).
288. Kaasik, K. & Lee, C. C. Reciprocal regulation of haem biosynthesis and the circadian clock in mammals. Nature 430, 467-71 (2004).
289. Zheng, B. et al. Nonredundant roles of the mPer1 and mPer2 genes in the mammalian circadian clock. Cell 105, 683-94 (2001).
290. Rudic, R. D. et al. BMAL1 and CLOCK, Two Essential Components of the Circadian Clock, Are Involved in Glucose Homeostasis. PLoS Biol 2, e377 (2004).
291. Fukuhara, C. Effect of dark exposure in the middle of the day on Period1, Period2, and arylalkylamine N-acetyltransferase mRNA levels in the rat suprachiasmatic nucleus and pineal gland. Brain Res Mol Brain Res 130, 109-14 (2004).
292. Chansard, M., Iwahana, E., Liang, J. & Fukuhara, C. Regulation of cAMP-induced arylalkylamine N-acetyltransferase, Period1, and MKP-1 gene expression by mitogen-activated protein kinases in the rat pineal gland. Brain Res Mol Brain Res 139, 333-40 (2005).
293. Izumo, M., Johnson, C. H. & Yamazaki, S. Circadian gene expression in mammalian fibroblasts revealed by real-time luminescence reporting: temperature compensation and damping. Proc Natl Acad Sci U S A 100, 16089-94 (2003).
294. Albrecht, U., Sun, Z. S., Eichele, G. & Lee, C. C. A differential response of two putative mammalian circadian regulators, mper1 and mper2, to light. Cell 91, 1055-64 (1997).
295. Jin, X. et al. A molecular mechanism regulating rhythmic output from the suprachiasmatic circadian clock. Cell 96, 57-68. (1999).
296. Pennisi, E. Multiple clocks keep time in fruit fly tissues. Science 278, 1560-1 (1997).
297. Plautz, J. D., Kaneko, M., Hall, J. C. & Kay, S. A. Independent photoreceptive circadian clocks throughout Drosophila. Science 278, 1632-5. (1997).
298. Plautz, J. D. et al. Quantitative analysis of Drosophila period gene transcription in living animals. J Biol Rhythms 12, 204-17 (1997).
299. Whitmore, D., Foulkes, N. S., Strahle, U. & Sassone-Corsi, P. Zebrafish Clock rhythmic expression reveals independent peripheral circadian oscillators. Nat Neurosci 1, 701-7 (1998).
300. Granda, T. G. et al. Circadian regulation of cell cycle and apoptosis proteins in mouse bone marrow and tumor. Faseb J 19, 304-6 (2005).
301. Ptitsyn, A. A. et al. Circadian Clocks Are Resounding in Peripheral Tissues. PLoS Comput Biol 2, e16 (2006).
302. Rutter, J., Reick, M. & McKnight, S. L. Metabolism and the control of circadian rhythms. Annu Rev Biochem 71, 307-31 (2002).
303. Young, M. E. The circadian clock within the heart: potential influence on myocardial gene expression, metabolism, and function. Am J Physiol Heart Circ Physiol 290, H1-16 (2006).
304. Schibler, U. Circadian rhythms. Liver regeneration clocks on. Science 302, 234-5 (2003).

305. Merrow, M. & Roenneberg, T. Cellular clocks: coupled circadian and cell division cycles. Curr Biol 14, R25-6 (2004).
306. Arjona, A. & Sarkar, D. K. Evidence supporting a circadian control of natural killer cell function. Brain Behav Immun (2005).
307. Shimba, S. et al. Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. Proc Natl Acad Sci U S A 102, 12071-6 (2005).
308. Lavery, D. J. et al. Circadian expression of the steroid 15 alpha-hydroxylase (Cyp2a4) and coumarin 7-hydroxylase (Cyp2a5) genes in mouse liver is regulated by the PAR leucine zipper transcription factor DBP. Mol Cell Biol 19, 6488-99 (1999).
309. Hirayama, J., Cardone, L., Doi, M. & Sassone-Corsi, P. Common pathways in circadian and cell cycle clocks: light-dependent activation of Fos/AP-1 in zebrafish controls CRY-1a and WEE-1. Proc Natl Acad Sci U S A 102, 10194-9 (2005).
310. Pregueiro, A. M., Liu, Q., Baker, C. L., Dunlap, J. C. & Loros, J. J. The Neurospora Checkpoint Kinase 2: A Regulatory Link Between the Circadian and Cell Cycles. Science (2006).
311. Myers, E. M. The circadian control of eclosion. Chronobiol Int 20, 775-94 (2003).
312. Myers, E. M., Yu, J. & Sehgal, A. Circadian control of eclosion: interaction between a central and peripheral clock in Drosophila melanogaster. Curr Biol 13, 526-33 (2003).
313. Tanoue, S., Krishnan, P., Krishnan, B., Dryer, S. E. & Hardin, P. E. Circadian clocks in antennal neurons are necessary and sufficient for olfaction rhythms in Drosophila. Curr Biol 14, 638-49 (2004).
314. Helfrich-Forster, C. Neurobiology of the fruit fly's circadian clock. Genes Brain Behav 4, 65-76 (2005).
315. Hastings, M. H. Circadian clocks: self-assembling oscillators? Curr Biol 13, R681-2 (2003).
316. Whitmore, D., Foulkes, N. S. & Sassone-Corsi, P. Light acts directly on organs and cells in culture to set the vertebrate circadian clock. Nature 404, 87-91. (2000).
317. Tamai, T. K., Carr, A. J. & Whitmore, D. Zebrafish circadian clocks: cells that see light. Biochem Soc Trans 33, 962-6 (2005).
318. Schibler, U. & Sassone-Corsi, P. A web of circadian pacemakers. Cell 111, 919-22. (2002).
319. Alvarez, J. D., Chen, D., Storer, E. & Sehgal, A. Non-cyclic and developmental stage-specific expression of circadian clock proteins during murine spermatogenesis. Biol Reprod 69, 81-91 (2003).
320. Morse, D., Cermakian, N., Brancorsini, S., Parvinen, M. & Sassone-Corsi, P. No circadian rhythms in testis: Period1 expression is clock independent and developmentally regulated in the mouse. Mol Endocrinol 17, 141-51 (2003).
321. Gachon, F., Nagoshi, E., Brown, S. A., Ripperger, J. & Schibler, U. The mammalian circadian timing system: from gene expression to physiology. Chromosoma (2004).
322. Filipski, E. et al. Persistent twenty-four hour changes in liver and bone marrow despite suprachiasmatic nuclei ablation in mice. Am J Physiol Regul Integr Comp Physiol (2004).
323. Manev, H. & Uz, T. Clock genes as a link between addiction and obesity. Eur J Hum Genet (2005).
324. Turek, F. W. et al. Obesity and Metabolic Syndrome in Circadian Clock Mutant Mice. Science (2005).
325. Durgan, D. J. et al. The circadian clock within the cardiomyocyte is essential for responsiveness of the heart to fatty acids. J Biol Chem (2006).
326. Reddy, A. B., Wong, G. K., O'Neill, J., Maywood, E. S. & Hastings, M. H. Circadian clocks: neural and peripheral pacemakers that impact upon the cell division cycle. Mutat Res 574, 76-91 (2005).
327. Klein, D. C. & Weller, J. L. Rapid light-induced decrease in pineal serotonin N-acetyltransferase activity. Science 177, 532-3 (1972).

328. Klein, D. C. et al. The melatonin rhythm-generating enzyme: molecular regulation of serotonin N-acetyltransferase in the pineal gland. Recent Prog Horm Res 52, 307-57; discussion 357-8 (1997).
329. Kennaway, D. J., Voultsios, A., Varcoe, T. J. & Moyer, R. W. Melatonin in mice: rhythms, response to light, adrenergic stimulation, and metabolism. Am J Physiol Regul Integr Comp Physiol 282, R358-65 (2002).
330. Weber, F., Hung, H. C., Maurer, C. & Kay, S. A. Second messenger and Ras/MAPK signalling pathways regulate CLOCK/CYCLE-dependent transcription. J Neurochem 98, 248-57 (2006).
331. Schibler, U., Ripperger, J. A. & Brown, S. A. Circadian rhythms. Chronobiology--reducing time. Science 293, 437-8 (2001).
332. Magnusson, I., Rothman, D. L., Katz, L. D., Shulman, R. G. & Shulman, G. I. Increased rate of gluconeogenesis in type II diabetes mellitus. A 13C nuclear magnetic resonance study. J Clin Invest 90, 1323-7 (1992).
333. Hundal, R. S. et al. Mechanism by which metformin reduces glucose production in type 2 diabetes. Diabetes 49, 2063-9 (2000).
334. Bray, G. A. Endocrine disturbances and reduced sympathetic activity in the development of obesity. Infusionstherapie 17, 124-30 (1990).
335. Nonogaki, K. New insights into sympathetic regulation of glucose and fat metabolism. Diabetologia 43, 533-49 (2000).
336. Qin, L. Q. et al. The effects of nocturnal life on endocrine circadian patterns in healthy adults. Life Sci 73, 2467-75 (2003).
337. Nicklas, T. A., Baranowski, T., Cullen, K. W. & Berenson, G. Eating patterns, dietary quality and obesity. J Am Coll Nutr 20, 599-608 (2001).

Summary

Because glucose is the essential fuel for the brain, glucose metabolism is tightly regulated in order to maintain plasma glucose levels within narrow limits. One of the strategies used by the organism to maintain glucose homeostasis is to adapt - or even predict - the glucose needs according to the time of the day. For instance, the anticipation to the daily activity period results in an increase in blood glucose levels just before the activity begins. The biological clock, located in the suprachiasmatic nucleus (SCN), is of vital importance for this temporal organization of the energy metabolism (of which glucose metabolism is one aspect). Indeed, previous studies have shown that the daily variations in plasma glucose concentrations and in glucose tolerance are driven by the SCN. Interestingly, the daily increase in plasma glucose levels coincides with a high glucose tolerance, indicating that just before awakening an increase of glucose output by the liver is required to compensate for the high glucose uptake. Furthermore, a recent study showed that the sympathetic input to the liver is essential for triggering an increase in plasma glucose levels (probably by an increase in hepatic glucose production), suggesting that the SCN, via the autonomic nervous system (ANS), influences liver metabolism in a circadian manner. In the current thesis, we therefore aimed at investigating the role of the ANS in the SCN-control of hepatic glucose metabolism according to the time of the day.

The rise in plasma glucose concentrations and the increased glucose uptake by peripheral tissues take place at the same time of the day: just before the onset of the activity period. The liver is the main organ for glucose production, therefore it has been suggested that the daily rise in plasma glucose levels might be attributed to an increase of hepatic glucose production. Although hormones such as glucagon, corticosterone, and growth hormone are known to stimulate hepatic glucose production, for various reasons they appear not to be involved in the control of the daily rhythm in plasma glucose concentrations. Recently it was shown that activation of the sympathetic input to the liver can trigger an increase in plasma glucose concentrations, which suggests a role of the autonomic route in the daily peak in plasma glucose levels. Here, we showed that, indeed, the sympathetic input to the liver is essential for driving the daily rhythm in plasma glucose levels, reinforcing our hypothesis that the SCN generates the daily rise in plasma glucose levels via a circadian control of hepatic glucose metabolism (Chapter 2). In line with this hypothesis, we also showed that the daily pattern in the gene expression of the enzymes involved in hepatic glucose metabolism (i.e., glucose-6-phosphatase, PEPCK, glucokinase, pyruvate kinase) was disturbed after the removal of the sympathetic innervation of the liver (Chapter 3).

Surprisingly, after a complete denervation of the ANS input to the liver in animals fed *ad libitum* (i.e., rhythmic feeding behavior), the plasma glucose levels maintained their daily rhythm, indicating that the ANS connection to the liver is not essential for the generation of the glucose rhythm (Chapter 3). Even more surprising, despite an a-rhythmic food intake (i.e., animals subjected to a 6-meal-schedule), liver-denervated animals still exhibit a 24h-rhythm in plasma glucose levels, indicating that the SCN uses another pathway to generate the daily rhythm in plasma glucose levels, for instance by the corticosterone rhythm (Chapter 3). In line with these data, we showed that the oscillatory patterns of the liver enzymes are also differentially controlled by the ANS (for the PEPCK), as well as by food intake (G6Pase and Glucokinase) and hormonal cues (for glycogen content). Taking all these observations together, we concluded that the SCN relies on more than one pathway to drive the hepatic glucose metabolism (i.e., liver enzymes) in order to generate a 24h-rhythm in plasma glucose levels. However, in case of a dysbalance of the autonomic output to the liver (created by just removing one autonomic branch of the liver), the liver metabolism is disturbed, which in turns results in an abolishment of the glucose rhythm.

A few years ago, it was demonstrated that clock genes (the molecular components of the clockwork) are not just located in the SCN, but throughout the entire organism. This discovery then led to the proposal of a new circadian timing system: the master clock in the SCN synchronizes, rather than drives, the peripheral clocks in order to generate a tissue-specific rhythmic output. In view of the circadian control of liver metabolism, we raised the hypothesis that the liver clock genes might play a role in the circadian expression of the liver enzymes and thereby drive a rhythmic glucose output (Chapters 2/6). However, our experimental approach did not yield a direct link between the oscillatory patterns of the clock genes and the liver enzymes, leading us to propose that the liver oscillator is not involved in the rhythmic hepatic glucose output.

Recent studies have shown that the inhibitory transmitter GABA mediates the light-induced inhibition of plasma melatonin levels via the sympathetic pre-autonomic neurons in the PVN. These observations led us to investigate further the role of the ANS and clock genes in the adjustment of the liver metabolism related to changes in environmental light conditions. Our first approach was to infuse a GABA-antagonist (known to block the inhibitory effect of light-induced GABA release on the pre-autonomic neurons in the PVN) in the PVN during the daytime. This experimental approach triggered an increase in plasma glucose levels, most likely by an increase of hepatic glucose production mediated via the sympathetic innervation of the liver (Chapter 4). Unfortunately, infusion of the GABA-antagonist in the PVN resulted in activation of all preautonomic neurons, i.e. increases in glucagon and corticosterone.

Therefore, despite hepatic sympathetic denervation, we were unable to identify a selective effect of the sympathetic input on the liver clock-genes and enzyme transcripts (Chapter 4). As a second approach, we decided to affect the autonomic output of the PVN in a more specific manner, by using a light pulse. We showed that a light pulse applied at night triggered a different physiological response according to the time at which light was applied: for instance, light exposure at CT14 stimulated corticosterone secretion, whereas light applied at CT20 did not (Chapter 5). However, the light-induced effect on the clock genes and functional genes did not indicate a role of the clock genes in the "physiological response" of the organs (Chapter 5), again suggesting that the peripheral clock genes might not have a role in the physiological response of the organs (Chapter 6). Finally, and more interestingly, we showed that also the light-induced changes in liver metabolism are mediated by the ANS (as shown previously for the pineal and the adrenal metabolism), and not by light-induced hormonal changes such as melatonin or corticosterone (Chapter 5).

In conclusion, the current thesis shows that the SCN influences hepatic glucose metabolism by using multiple mechanisms (e.g., ANS, food intake, hormonal cues). Furthermore, a balanced autonomic input to the liver is required to maintain a rhythmic pattern of liver enzymes and thereby the 24h-rhythm in plasma glucose levels. When the light conditions change, the SCN is able to influence the peripheral metabolism immediately, by using the autonomic innervation of the organs, such as the pineal and the adrenal, but also the liver.

Résumé

Le glucose est le nutriment utilisé comme source énergétique par tous les tissues. Bien que les apports du glucose puissent varier au cours du temps (par l'alimentation), la glycémie (concentration du glucose dans le sang) reste toujours comprise entre 4.5-6.5 mmol/l. Le maintien de cette concentration en glucose à l'intérieur de limites strictes en dépit des contraintes extérieures est important pour le bon fonctionnement de l'organisme. Le processus mis en jeu pour conserver cette valeur plus ou moins constante est appelé homéostasie. La régulation précise de la glycémie dépend d'un équilibre entre la production/consommation (ou stockage) de glucose. En cas d'hyperglycémie, le glucose pénètre dans les organes tels que les reins, les muscles et surtout le foie pour être stocké sous forme de glycogène (glycogenèse). En cas d'hypoglycémie, le glucose est principalement libéré dans le sang par le foie (glycogénolyse et/ou gluconéogenèse). L'équilibre entre la production et l'utilisation de glucose dépend de plusieurs tissues et est sous le contrôle d'hormones pancréatiques (insuline et le glucagon) et du système nerveux autonome. En plus des mécanismes qui répondent de manière immédiate aux changements abrupts de glycémie, l'efficacité du maintien de l'homéostasie repose également sur la capacité de l'organisme à anticiper les variations extérieures. Ce phénomène d'anticipation, appelé « dawn phenomenon », a été décrit chez l'homme (1984) et les rongeurs (1967), chez qui il a été montré que la concentration en glucose dans le plasma augmente juste avant le début de la période active (tôt le matin pour l'homme, en fin de journée pour les rongeurs à activité nocturne). Ce phénomène d'anticipation repose sur une structure, les noyaux suprachiasmatiques NSC, localisée dans l'hypothalamus qui est communément appelé l'horloge biologique ou circadienne. Dans ce concept les NSC organisent les paramètres physiologiques et métaboliques en fonction du cycle jour/nuit telle qu'une augmentation de la glycémie, de la concentration de corticostérone dans le sang et de la température corporelle juste avant le réveil. Cette organisation temporelle du métabolisme et des fonctions physiologiques est un facteur essentiel pour le maintien de la vie des organismes. Jusqu'à maintenant peu d'attention a été portée sur le rôle du système nerveux autonome (SNA) dans la régulation de l'homéostasie du glucose et surtout son rôle dans le phénomène d'anticipation des besoins en glucose juste avant la période active. Dans notre travail, nous nous sommes principalement attaché à répondre à la question de savoir comment l'horloge circadienne contrôle/régule l'homéostasie du glucose, avec un intérêt particulier sur le rôle du système nerveux autonome. Notre hypothèse était que le message journalier délivré par l'horloge est intégré par les oscillateurs, localisés dans les organes périphériques (foie), afin de synchroniser la fonctionnalité de chaque organe avec le cycle jour/nuit (homéostasie du glucose).

Les variations journalières des concentrations de glucose dans le plasma présentent un rythme circadien caractérisé par un pic juste avant le début de la période d'activité (i.e., la nuit pour les rongeurs). Pendant longtemps, il a été admis que le profil rythmique de la glycémie était une conséquence de la rythmicité de la prise alimentaire. Cependant, lorsque les rats sont entrainés à un régime alimentaire arythmique (i.e., accès à la nourriture tous les 4 heures), le rythme de glycémie reste inchangé ce qui indique un rôle non pas de la prise alimentaire mais de l'horloge. Celle-ci serait responsable de la rythmicité de la concentration de glucose dans le plasma. La question était donc de savoir comment l'horloge génère les variations journalières de la glycémie. Comme il a été démontré que cette augmentation du glucose dans le sang coincide également à un moment de la journée où la tolérance au glucose par les organes périphérique est élevée, cette hyperglycémie ne pouvaient être expliquée que par une production de glucose au même moment par le foie (principale source de glucose). Pour générer cette hyperglycémie journalière, juste avant la période active, les NSC peuvent agir sur le métabolisme du foie par deux principales voies : la voie hormonale (avec des hormones hyperglycémiante tel que le glucagon ou la corticostérone) et la voie nerveuse (les deux branches du SNA). Pour des raisons diverses, il est improbable que la voie hormonale soit responsable de cette hyperglycemie journaliere. Récemment nous avons démontré que l'activation du nerf sympathique induit une hyperglycémie (Chapitre 4), c'est pour ces raisons que nous avons alors proposé que les NSC via le nerf sympathique participent à la genèse du profil rythmique des concentrations de glucose dans le sang, en induisant une augmentation de la glycémie juste avant la nuit. De plus, nous avons travaillé sur l'hypothèse que ces variations de la glycémie résultaient d'un contrôle de l'horloge sur la fonctionnalité du foie, via les gènes horloges localisées dans les cellules de cet organe (i.e., les gènes horloges génèreraient la rythmicité des enzymes impliquées dans le métabolisme du glucose).

Nous avons effectivement démontré qu'une lésion sélective du nerf sympathique au niveau du foie abolit le rythme du glucose dans le sang, confirmant que le système nerveux autonome (ici specifiquement le nerf sympathique) est essentiel pour générer les variations journalières de la glycémie. De plus, cette déinnervation sélective du foie affecte également l'expression rythmique des enzymes hépatiques, contrairement à l'expression des gènes horloges. Ces résultats nous permettent de conclure que les NSC, via les SNA, controle le métabolisme du glucose dans le foie pour générer le rythme du glucose. Cependant l'oscillateur dans le foie ne semble pas directement impliqué dans la genèse du profil circadien de la glycémie (Chapitre 2).

Dû à l'absence de relation entre la rythmicité des gènes horloges et des enzymes impliquées dans le métabolisme du glucose (voir également Chapitre 6), nous nous sommes alors focalisés sur l'étude du rôle du SNA et de la prise alimentaire dans le maintien de l'expression rythmique des enzymes hépatiques. Nous avons étudié

l'expression des enzymes et la concentration du glucose dans le plasma sur 24h après une dénervation complète du foie (nerf sympathique et parasympathique) chez des animaux placés en condition *ad libitum* ou en régime alimentaire arythmique. Avec ces deux approches, nous avons pu identifier les gènes dont la transcription rythmique est régulé par le système nerveux autonome (Pepck) de ceux régulés par la prise alimentaire (G6Pase et Glucokinase). En absence de connexion hypothalamus-foie (via le SNA) la rythmicité de la glycémie est maintenue chez les animaux placés en condition *ad libitum*, indiquant que la prise alimentaire participe également à la genèse de la rythmicité du glucose. En absence de signal rythmique fournit soit par le SNA soit par une prise alimentaire, il est apparu que le rythme de 24h dans les concentrations plasmatiques de glucose était toujours présent. Cela indique qu'un autre signal rythmique contribue au rythme du glucose (la corticostérone ?) (Chapitre 3).

Durant le jour (ou jour subjectif en conditions constantes), les NSC envoient un message inhibiteur (par la sécrétion du neurotransmetteur GABA) aux noyaux paraventriculaires de l'hypothalamus (PVN) qui provoque l'inactivation des neurones pré-autonomiques du système sympathique et parasympathique. Récemment, dans notre groupe de recherche il a été démontré que le blocage de ce message inhibiteur, par une infusion d'un antagoniste (bicuculline) de GABA au niveau des PVN, provoque l'activation des neurones pré-autonomiques et induit une augmentation de la glycémie. Bien que l'infusion de bicuculline induit une activation de tous les neurones dans les PVN et par conséquent une augmentation de corticostérone et de glucagon dans le sang (hormones hyperglycémiantes), cette augmentation de glucose dans le sang est complètement absente après une lésion du nerf sympathique spécifique au foie. Nous avons alors proposé que cette stimulation du nerf sympathique affecte l'expression des gènes horloges dans le foie ce qui, en retour, va activer la production/libération de glucose (par le foie) aboutissant alors à une hyperglycémie. Pour valider cette hypothèse, nous avons analysé dans le foie, l'expression des principaux gènes horloges (Per1/2/3, Cry1/2 et DBP) ainsi que l'expression des enzymes impliquées dans le métabolisme du glucose tels que Glucose-6-phosphatase (enzyme qui permet la libération du glucose dans le sang), Glucokinase (enzyme permettant au glucose en excès dans le sang de pénètrer dans le foie pour ensuite être stocké sous forme de glycogène), Pepck (enzyme clé dans la production de glucose par Gluconéogenèse). Cette stimulation massive des neurones localisés dans les PVN nous a permis de ne définir que partiellement l'effet spécifique du nerf sympathique du foie sur l'expression des gènes horloges comme sur les enzymes impliquées dans l'hyperglycémie (Chapitre 4). Nous avons alors changé notre approche expérimentale, en examinant l'activation, par un signal lumineux pendant la nuit, de la voie multisynaptique NSC-PVN-SNA-foie sur le métabolisme du glucose (Chapitre 5). Nous avons pu ainsi observer que l'application d'un signal lumineux d'une heure en début de nuit et/ou en fin de nuit induisent un effet immé-

diat sur l'expression génique de certains gènes horloges dans different organes étudiés mais également sur certaines enzymes impliquées dans le métabolisme, tel que Pepck et G6Pase pour le foie, NAT pour la glande pinéal. Cependant aucun lien direct n'a pu être démontré entre l'expression des gènes horloges et des gènes functionnelles en réponse à l'application d'un créneau lumineux. L'application d'un créneau lumineux en début de nuit (CT 14), nous a permis dans un premier temps de démontrer que cette information lumineuse agit directement sur la physiologie des organes comme la glande pinéal (i.e., stopper la production de la mélatonine), la glande surrénale (induire la sécrétion de corticostérone) et aussi le foie. De plus, nous avons pu demontre, pour la premiere fois, que cette information lumineuse, percue par les NSC, est directement transmise au foie via le SNA et non pas de manière indirect par une diminution de la mélatonine et/ou une sécrétion de corticostérone.

Pour conclure, l'horloge circadienne peut ajuster/modifier rapidement le métabolisme du foie via le SNA et ce, par rapport aux changements du cycle jour/nuit. Ce mécanisme de « resynchronisation » ne semble pas mettre en jeu l'oscillateur du foie. Sur 24 heures, la genèse du rythme circadien des concentrations plasmatiques de glucose repose sur plusieurs mécanismes, le SNA, la rythmicité de la prise alimentaire et du profile journalier de la concentration de corticostérone dans le plasma. Cependant, un déséquilibre dans le système nerveux autonome (induit par exemple par une dénervation d'une seule branche du SNA) cause une anormalité dans le fonctionnement hépatique ce qui résulte une perte de la rythmicité du glucose.

Samenvatting

Glucose is van essentieel belang als brandstof voor de hersenen en glucose metabolisme is derhalve strict gereguleerd, zodat de plasma glucosespiegels binnen nauwe grenzen blijven. Een van de strategieën die het organisme gebruikt om glucose homeostase in stand te houden is het aanpassen - en zelfs het voorspellen - van de glucosebehoefte naar gelang het uur van de dag. Zo resulteert de anticipatie op de activiteitsperiode bijvoorbeeld in een stijging van de glucosespiegels in het bloed reeds voordat de activiteit begint. De biologische klok, die zich bevindt in de hypothalame suprachiasmatische nucleus (SCN), is van essentieel belang voor de temporele organisatie van het energiemetabolisme (zoals het glucose metabolisme). Eerdere studies hebben inderdaad al aangetoond dat de dagelijkse schommelingen in de plasma glucoseconcentratie en in glucosetolerantie worden bewerkstelligd door de SCN.

Het is interessant dat de dagelijkse verhoging van de plasma glucosespiegels samenvalt met een hogere glucosetolerantie; een indicatie dat reeds voor het ontwaken een verhoging van de glucoseproductie nodig is om de aanstaande hoge glucose-opname te compenseren. Verder laat recent onderzoek zien dat de sympatische input naar de lever essentieel is voor het verhogen van de plasma glucosespiegels (waarschijnlijk door middel van een verhoging van de glucoseproductie door de lever), hetgeen er op wijst dat de SCN, via het autonome zenuwstelsel (ANS), het levermetabolisme mogelijk op circadiane wijze beïnvloedt. Dit proefschrift beoogt dan ook te onderzoeken welke rol het ANS speelt in de dagelijkse controle van de SCN over het (glucose)metabolisme in de lever.

Tijdens de slaap/waak-cyclus vinden verhoogde plasma glucoseconcentraties en een verhoogde glucose-opname door perifere weefsels gelijktijdig plaats, te weten vlak voor het begin van de activiteitsperiode. Daarom wordt dan ook gedacht dat op hetzelfde moment ook de glucoseproductie verhoogd is. De lever is het belangrijkste orgaan voor de glucoseproductie. Hoewel bekend is dat hormonen zoals glucagon, corticosteron en groeihormoon glucoseproductie door de lever kunnen stimuleren zijn ze om verschillende redenen waarschijnlijk toch niet betrokken bij de regulatie van het dag/nacht-ritme in de plasma glucoseconcentratie. Recentelijk is aangetoond dat activering van de sympathische input naar de lever een verhoging van plasma glucoseconcentraties teweeg kan brengen, hetgeen er op duidt dat de autonome route een rol speelt in de dagelijkse piek van glucosespiegels.

In Hoofdstuk 1 laten we zien dat de sympatische input naar de lever inderdaad essentieel is voor het in stand houden van het dag/nacht-ritme in plasma glucosespiegels. Dit ondersteunt onze hypothese dat de SCN de dagelijkse stijging van plasma

glucosespiegels genereert, door middel van veranderingen in de hepatische glucoseproductie.

Overeenkomstig met deze hypothese hebben we tevens aangetoond dat het 24-uurs patroon van de genexpressie van de enzymen betrokken bij het hepatisch glucosemetabolisme (i.e. glucose-6-phosphatase, PEPCK, glucokinase, pyruvate kinase) verstoord raakte na verwijdering van de sympatische innervatie van de lever (Hoofdstuk 2).

Na een totale denervatie van de ANS input naar de lever in dieren die *ad libitum* te eten hadden (d.w.z. ritmisch eetgedrag vertoonden) bleek het dag/nacht-ritme van de plasma glucosespiegels verrassend genoeg in stand gebleven te zijn, wat aangeeft dat de connectie van het ANS met de lever niet essentieel is voor het genereren van het glucose ritme (Hoofdstuk 2). Nog verrassender was dat ook dieren met een a-ritmische voedselopname (d.w.z. onderworpen aan een 6-maaltijden schema), en een totale lever denervatie, toch nog een 24-uurs ritme hadden in hun plasma glucosespiegels, wat aangeeft dat de SCN een andere route gebruikt om het dag/nacht-ritme van plasma glucosespiegels te genereren, bijvoorbeeld het corticosteronritme (Hoofdstuk 2).

Overeenkomstig met deze gegevens hebben we tevens aangetoond dat het oscillatiepatroon van de leverenzymen onder differentieel beheer staat van het ANS (wat betreft PEPCK), voedselopname (G6Pase en glucokinase) en hormonale prikkels (voor de hoeveelheid glycogeen). Al deze observaties tesamen brachten ons tot de conclusie dat de SCN op meer dan één pad vertrouwt als het gaat om het aansturen van het hepatische glucosemetabolisme voor het genereren van een 24-uurs ritme in de plasma glucosespiegels. Echter, een onbalans van de autonome output naar de lever (veroorzaakt door het wegnemen van één van de twee autonome inputs naar de lever), is al voldoende om het levermetabolisme te verstoren, en resulteert in het verdwijnen van het glucoseritme.

Een aantal jaren geleden is aangetoond dat klokgenen (een onderdeel van het moleculaire uurwerk) niet alleen in de SCN gevonden geworden maar door het gehele organisme heen. Deze ontdekking leidde vervolgens tot een herziening van de ideeën over de opbouw van het circadiane systeem: de SCN, de hoofdklok, synchroniseert de perifere klokken, i.p.v. deze aan te sturen, en deze perifere klokken genereren vervolgens hun eigen weefsel-specifieke ritmische output resulterend in een ritmische activiteit van het orgaan. Er van uitgaande dat het levermetabolisme circadiaan geregeld wordt veronderstelden wij dat de klokgenen in de lever een rol spelen bij de circadiane expressie van de leverenzymen en daarmee een ritmische glucose output aansturen (Hoofdstuk 1 en 6). Echter, onze resultaten leverden geen bewijs voor een directe relatie tussen de oscillatiepatronen van de klokgenen en het leverenzym, hetgeen leidde tot ons voorstel dat een ritmisch activiteit van de leveroscillator niet voldoende is voor een ritmische hepatische glucoseproductie.

Recent onderzoek heeft aangetoond dat de inhibiterende transmitter GABA de lichtgeïnduceerde inhibitie van de plasma melatoninespiegels medieert via de sympathische pre-autonome neuronen in de PVN. Deze observaties hebben er toe geleid dat wij de rol van het ANS en klokgenen bij het aanpassen van het levermetabolisme bij veranderingen in omgevingslicht nader zijn gaan bestuderen. Onze eerste benadering was om gedurende de lichtperiode een GABA-antagonist (waarvan we weten dat het het inhiberende effect van licht (d.m.v. GABA), op de pre-autonome neuronen in the PVN, blokkeert) toe te dienen in de PVN (Hoofstuk 4). Dit experiment leidde tot een verhoging van de plasma glucosespiegels (waarschijnlijk door een verhoging van de hepatische glucoseproductie via de sympatische innervatie van de lever). Helaas resulteerde de infusie van de GABA-antagonist in de PVN in een activatie van alle pre-autonome neuronen (verhoogde glucagon- en corticosteronspiegels), hetgeen betekende dat we niet in staat waren een selectief effect van de sympatische input op de klokgenen van de lever en enzymtranscripten waar te nemen (Hoofdstuk 4). Onze tweede benadering was de autonome output van de PVN meer specifiek te stimuleren, door middel van een lichtpuls. We toonden aan dat een nachtelijke lichtpuls een andere fysiologische respons opriep, al naargelang het tijdstip waarop de puls plaatsvond: bijvoorbeeld, blootstelling aan licht aan het begin van de nacht stimuleerde de afgifte van corticosteron, terwijl dat niet het geval was aan het eind van de nacht (Hoofdstuk 5). Verder liet het door licht geïnduceerde effect op de klokgenen en op de functionele genen geen rol zien voor de klokgenen voor wat betreft de 'fysiologische respons' van de organen (Hoofdstuk 5), een extra aanwijzing dat de klokgenen uit de periferie wellicht geen rol spelen in de fysiologische respons van de organen (Hoofdstuk 6). Lest best: we hebben aangetoond dat de door licht geïnduceerde veranderingen in het lever metabolisme worden overgebracht door het ANS (zoals eerder reeds was aangetoond voor het epifyse- en bijniermetabolisme) en niet via licht geïnduceerde hormonale veranderingen zoals melatonine of corticosteron (Hoofdstuk 5).

Concluderend kunnen we zeggen dat dit proefschrift laat zien dat de SCN het glucosemetabolisme in de lever beïnvloedt door middel van diverse mechanismen (b.v. ANS, voedselopname, hormonale prikkels). Voorts, dat een evenwichtige autonome input in de lever vereist is voor de handhaving van het ritmische patroon van de leverenzymen - en daarmee van het 24-uursritme van de plasma glucosespiegels. Wanneer de lichtcondities veranderen is de SCN in staat om onmiddellijk het periferie-metabolisme te beïnvloeden middels zijn contacten met de autonome innervatie van de organen, zoals de epifyse en de bijnier, maar ook van de lever.

Publications

Poirel VJ, Cailotto C, Streicher D, Pevet P, Masson-Pevet M, Gauer F, (2003) MT1 melatonin receptor mRNA tissular localization by PCR amplification, *Neuro Endocrinol Lett*.;24(1-2) :33-8.

Kamphuis W, Cailotto C, Dijk F, Bergen A, Buijs RM (2005). Circadian expression of clock genes and clock-controlled genes in the rat retina. *Biochem Biophys Res Commun*.;330(1):18-26.

Cailotto C, La Fleur S.E, van Heijningen C, Wortel J, Kalsbeek A, Feenstra M, Pévet P, and Buijs RM (2005).The suprachiasmatic nucleus controls the daily variation of plasma glucose via the autonomic output to the liver: are the clock genes involved? *European Journal of Neurosciences*;22(10):2531-40.

Kalsbeek A., Ruiter M., La Fleur S.E, Cailotto C., Kreier F. and Buijs RM (2006). Hypothalamic control of Glucose Homeostasis. *Progress in Brain Research*, Vol 153, Hypothalamic integration of Energy metabolism, Elsevier, Amsterdam, pp. 283-307.

Kalsbeek A, Palm I.F, La Fleur S.E, Scheer F.A.J.L, Perreau-Lenz S, Ruiter M, Kreier F, Cailotto C and Buijs RM, SCN Outputs and the Hypothalamic Balance of Life, Special issue of Journal of Biological Rhythms on Expression of Circadian Rhythms *In press, December 2006.*

Cailotto C, van Heijningen C, van der Vliet J, van der Plasse G, Kalsbeek A, Pévet P, Buijs RM. Daily rhythms in metabolic liver enzymes and plasma glucose require a balance in the autonomic output to the liver. *Submitted*

Cailotto C, van Heijningen C, van der Vliet J, Pévet P, Kalsbeek A and Buijs RM. PVN stimulation affects clock gene and glucose-metabolizing enzyme expression in the liver: Does the SCN use the autonomic nervous system to drive liver metabolism in a circadian manner? *Submitted*

Cailotto C, Lei J, van Heijningen C, van der Vliet J, van Eden C, Kalsbeek A., Pévet P, Buijs RM. Light affects the functionality of organs via the autonomic nervous system. *In preparation.*

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Curriculum Vitae

Cathy Cailotto was born in Metz, France, in February 1976. In 1994 she graduated from the *Lycée Robert Schuman* of Metz in France.

She studied biology at the *Université Paul Verlaine* of Metz for three years and went on to the *Université Louis Pasteur* of Strasbourg to specialize in Neurosciences. She obtained a Master's degree in Neurosciences in July 2000.

During these studies, she performed two practical training courses on electrophysiology: 'Patch Clamp, whole cell configuration' and 'Patch Clamp voltage dependent' in two laboratories (UMR CNRS/ ULP 7168 LC2) in Strasbourg. Another molecular biology practical training course, *in situ* hybridization, was carried out at the laboratory *Neurobiologie des Rythmes* (UMR CNRS/ ULP 7168/LC2).

Her first research training period was at the laboratory *Neurobiologie des Rythmes* in Strasbourg under the supervision of Dr. Mireille Masson-Pévet and Prof. Dr. François Gauer. She studied the role of the Mt1 receptor sub-type in the melatonin effects on the SCN in Siberian Hamsters. The technical approach was to block the production of the Mt1 receptors at the level of its mRNA by infusion antisense oligonucleotides in the SCN.

After obtaining her Master of Science degree, she took the opportunity to do a co-supervised PhD with two important groups in the field of biological rhythms, the group of Prof. Dr. Paul Pévet in Strasbourg and the group of Prof. Dr. Ruud Buijs in the *Netherlands Institute for Neuroscience* in Amsterdam, which she began on September 3, 2001. Under the supervision of Dr. Andries Kalsbeek, Prof. Dr. Paul Pévet and Prof. Dr. Ruud Buijs, she extended her knowledge on how the biological clock organizes daily physiology. The results of this training period are presented in this thesis.