Biological clock control of daily glucose metabolism: hormonal and autonomic pathways

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CHAPTER VI

General Discussion

Based on
Hormones and the autonomic nervous system are involved in suprachiasmatic nucleus modulation of glucose homeostasis.
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Circadian control of glucose homeostasis
Because it is essential for survival that glucose is available to the brain at all times, glucose metabolism is regulated in a very strict way. This involves several hormonal and neuronal pathways with overlapping and supplementary functions.

Many aspects of glucose metabolism were shown to be controlled by the SCN, first by Nagai and coworkers, who stimulated the SCN electrically. This resulted in augmented plasma glucose concentrations\(^{193}\), an effect that could be prevented by the peripheral administration of α- and β-blockers\(^{194}\). The aim of our studies was to identify the pathways and mechanisms by which the SCN controls the different aspects of glucose metabolism, responsible for the daily rhythm in basal plasma glucose concentrations as described previously\(^{201}\). The studies presented in this thesis indicate that the SCN mainly uses neuronal pathways to control the basal rhythm in plasma glucose concentrations. Furthermore, we propose that three subdivisions can be identified in the mechanisms through which the SCN modulates daily (glucose) homeostasis. First, the clock prepares the body for an upcoming period of activity or rest by ‘anticipatory’ homeostasis. Second, counterregulatory responses to acute disturbances of the internal milieu are adapted to the moment of the day. Third, in order to use available nutrients as efficiently as possible, the clock modulation of physiology and energy expenditure is enhanced during times of famine. The results of our studies are discussed according to this subdivision.

Anticipatory control of homeostasis
Glucose production – hormonal control
Both plasma glucose concentrations\(^{201,204}\) and glucose disposal\(^{202,205-210}\) are high at the onset of the activity period. Consequently, it has been suggested that hepatic glucose production is stimulated at the onset of the activity period\(^{203,204,211}\).
Glucose production is stimulated by the pancreatic hormone glucagon. Many studies have shown a daily rhythm in the basal plasma concentrations of this glucogenic hormone. However, the results of these studies were inconsistent with respect to phase and amplitude of the rhythm. Data from our own study (chapter 2) show that glucagon indeed displays a daily rhythm entrained by the SCN. However, this rhythm is not directly correlated to the daily glucose rhythm, i.e. peak glucagon concentrations do not coincide with peak glucose concentrations. Furthermore, we showed that glucagon release responded very strongly to the feeding episodes in a scheduled feeding regimen, whereas the daily glucose rhythm is independent of this (scheduled feeding) regimen.

Fig. 1 The suprachiasmatic nucleus (SCN) sends its signal of day and night to the rest of the body via several different pathways. Both "parasympathetic" (light gray) and "sympathetic" (dark grey) neurons project to cells in the PVN (and other hypothalamic nuclei involved in glucose metabolism). The pre-autonomic PVN cells project to the dorsal motor nucleus of the vagus (DMV, parasympathetic) in the brainstem and the intermediolateral column of the spinal cord (IML, sympathetic) that relay the signal to organs in the periphery. Furthermore, the pituitary receives the SCN signal through the release of "releasing factors" from the PVN and rhythmically secretes hormones that may have an effect on glucose production. Peripheral feedback signals concerning glucose availability, energy reserves in the body and hormone levels are received by the hypothalamus via different sensory pathways. (From Buijs et al. 2001 with permission)
Other hormones that stimulate glucose production also display daily rhythms in their plasma concentrations. Growth hormone (GH) promotes hepatic glucose output\textsuperscript{164} and has been suggested to play a role in the dawn phenomenon in humans\textsuperscript{386}. Rats, however, show an ultradian rather than circadian pattern of GH release\textsuperscript{223,224,387}, which makes a significant role for GH in the dawn phenomenon unlikely, at least in rats.

Corticosteroids enhance glucose production\textsuperscript{165}, and their daily rhythms\textsuperscript{215,218,220,386,221} correlate very well with the glucose pattern observed in mammals\textsuperscript{201,204,388}. It would be a good candidate for regulating this glucose pattern\textsuperscript{209,390}. However, a rise in blood glucose levels still occurs if the morning rise of cortisol levels in humans is prevented\textsuperscript{222,391}, which makes a major role for corticosteroids in the modulation of daily glucose metabolism unlikely.

Epinephrine also stimulates glucose production and is released from the adrenal medulla in a circadian release pattern in humans\textsuperscript{392,393}, but is not a likely candidate to explain the basal glucose profile. Its peak occurs somewhat later than the peak in plasma glucose concentrations\textsuperscript{201,204}, and in rats, daily epinephrine and norepinephrine profiles are mainly related to the locomotor activity pattern\textsuperscript{219}, whereas the glucose profile is not\textsuperscript{201}. Taken together, it is unlikely that hormones play a major role in daily variations of glucose production. Rather, the SCN affects glucose production via neuronal pathways.

**Glucose production – neuronal control**

The anatomical basis for a possible SCN control of glucose production via neuronal projections to glucose-producing organs has been shown by viral tracing studies. Virus-infected neurons after injection of pseudorabies virus in the liver were shown in several hypothalamic areas, such as the PVN, LHA, MPO, a small number of VMH neurons, and the SCN\textsuperscript{102}. Injection into the kidneys revealed that its autonomic innervation also originates in these hypothalamic areas\textsuperscript{196}. The small intestine also produces glucose, but the presence of autonomic projections from the SCN has not been investigated.

There is also functional evidence of SCN control of glucose production via the ANS. Silencing the SCN or DMH (a target area of the SCN\textsuperscript{12,27}) with tetrodotoxin (TTX) increases HGP\textsuperscript{198}. Furthermore, activation of the PVN (another important SCN target area\textsuperscript{12,258,308,347,351,394,395}), by administration of GABA-antagonists or glutamate agonists\textsuperscript{197,198}, results in increased HGP. Denervation of the sympathetic hepatic branch of the ANS prevents this increase\textsuperscript{198}. Furthermore, also the arcuate nucleus and preoptic area may be involved in SCN control of hepatic glucose output. Both receive SCN projections\textsuperscript{12}, project to the PVN\textsuperscript{396,397} and are involved in glucose homeostasis\textsuperscript{355,364,398}. 

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We propose that the SCN inhibits glucose output during the resting period, via an inhibition of the pre-autonomic neurons in the PVN that are stimulating to the sympathetic outflow to the liver. Probably, HGP increases towards the onset of the activity period because the inhibition of the sympathetic output to the liver is gradually removed. We suggest that the SCN modulates the balance between sympathetic and parasympathetic output to the liver, although at present there is no clear evidence for a physiological function of the described SCN control of the parasympathetic outflow to the liver. The explanation might be that an important part of the parasympathetic preautonomic neurons reside in the DMH. A similar mechanism of sympathetic and parasympathetic outflow may be employed for SCN control of the pancreas, at least in fasted animals. This will be explained below.

Glucose disposal

Previous studies in our group have shown that the SCN modulates glucose uptake in peripheral tissue. Glucose uptake can be stimulated via neural pathways, as described in Chapter 1, and we suggested that the SCN may control glucose uptake in the basal situation. Furthermore, we hypothesized that glucose uptake in peripheral organs may be regulated differentially, depending on their different functionalities and different control by the CNS. A third hypothesis was that a daily rhythm in insulin-inhibited glucose production may have affected the plasma glucose concentrations measured during glucose tolerance test, and explain the daily variations in the glucose peaks induced by the glucose bolus. In order to examine whether time dependent and tissue dependent differences in glucose uptake could be demonstrated, we used 2-deoxy-	extsuperscript{3}H-D-glucose (H-2DG), a type of glucose that cannot be metabolized, to assess glucose uptake at different moments of the L/D cycle. We were not able to show a daily rhythm in basal glucose uptake in the tissues that were analyzed.

Possibly, a daily rhythm in glucose tolerance is due to an insulin-induced inhibition of hepatic glucose production (HGP), which may result in lower blood glucose concentrations that could be mistaken for increased glucose tolerance. However, our data suggest that it is uncertain whether the H-2DG technique is an adequate tool for measuring glucose uptake in vivo in basal conditions, although it has been used in many studies in combination with a hyperinsulinemic clamp. Recently, however, the presence of glucose-6-phosphatase (G6Pase), the enzyme that de-phosphorylates glucose or H-2DG and allows it to be released from the cell, has been shown in skeletal muscle, although it was previously presumed that G6Pase is only active in the liver, kidneys and intestine. The authors have assessed G6Pase activity in vitro, but not yet in vivo. Our data indicated that H-2DG does not accumulate in the tissue, suggesting that H-2DG may indeed be released instead of being trapped in the cell. Because we did not use a hyperinsulinemic clamp, a lack of high insulin
concentrations may have prevented the balance between $^3$H-2DG uptake and release to shift towards uptake. Possibly, the $^3$H-2DG technique is not suitable for measuring glucose uptake in the basal situation (i.e. without hyperinsulinemic clamp). To our knowledge, the $^3$H-2DG technique has been used in one in vitro study to provide evidence for a rhythm in both basal and insulin-stimulated glucose uptake in skeletal muscle cell and adipocyte cultures. These cells were cultured for over 1 week before uptake was assessed. Therefore, differences are caused entirely by differences in clock gene expression and not by direct SCN control. Based on these data we propose that also in vivo a daily rhythm in basal glucose uptake might indeed exist. Furthermore, we suggested a differential SCN control of glucose uptake, i.e. different tissues may have different daily rhythms in glucose uptake. However, due to the technical issues described here we have not been able to test these hypotheses. To clarify this, clamp studies are needed to show which tissues account for glucose uptake during glucose or insulin tolerance tests. However, for the analysis of basal glucose uptake, a hyperinsulinemic clamp is not suitable.

**Glucose disposal – interaction with lipid metabolism**
Because lipid and glucose metabolism are in close interaction, it has been suggested that rhythmic properties of lipid metabolism may have an impact on (daily) glucose metabolism, i.e. high plasma glucose concentrations may be due to high FFA concentrations that inhibit peripheral glucose uptake. Daily rhythms in plasma FFA and triglyceride concentrations as well as lipogenic enzymes have been shown. However, we demonstrated that it is not likely that the daily rhythm in plasma glucose concentrations is the consequence of the daily changes in plasma FFA, because the daily plasma FFA profile does not match the daily plasma glucose profile (chapter 4). Nevertheless, we did provide further insight in the mechanisms of SCN control of lipolysis. Rats with SCN lesions had increased plasma FFA concentrations, which did not respond to fasting. These results suggested that the SCN inhibits lipolysis. We investigated whether this may be mediated via the PVN, since stimulation of the PVN also increases hepatic glucose production. Furthermore, it is well known that the PVN projects to white adipose tissue (WAT). Activation of the PVN, however, did not result in increased plasma FFA concentrations. Other studies have indicated that the MPO may be involved in lipid mobilization. Because the MPO also receives SCN projections, we propose that the SCN modulates lipid metabolism via the MPO rather than via the PVN.

Leptin and adiponectin both affect glucose metabolism and show a pronounced circadian rhythm. In the rat, peak leptin concentrations occur around the onset of the activity period. By contrast, the diurnal leptin profile in humans shows an
opposite pattern\textsuperscript{404-406}. The peak in plasma glucose concentrations occurs at the onset of the activity period in both humans and rats\textsuperscript{201,204}. \textit{In vivo} leptin administration stimulates glucose uptake in insulin sensitive tissues\textsuperscript{407,408}, whereas it inhibits glucose uptake in adipocytes and skeletal muscle fibers \textit{in vitro}\textsuperscript{409,410}. This indicates that leptin effects on glucose disposal are mediated via the central nervous system. The only rhythm that the daily leptin pattern correlates with in both humans and rats is the daily melatonin rhythm, i.e. peak levels occur during the dark period in both species\textsuperscript{411}. This would suggest that the rhythmic leptin release is due the presence of light rather than e.g. daily feeding or activity rhythms. We propose that it is unlikely that the daily leptin rhythm modulates the daily glucose rhythm directly.

Plasma adiponectin concentrations show a diurnal pattern in humans, with high levels during the day and a nadir during the sleeping period\textsuperscript{406}. Although the adiponectin pattern coincides more or less with the daily rhythm in insulin sensitivity, to our knowledge, a direct relation between the daily adiponectin and insulin sensitivity has never been shown.

\textbf{Summary}

We conclude that hormones and other nutrients like FFAs are not responsible for the daily changes in basal glucose metabolism. Daily rhythms in hormones that affect glucose metabolism, such as insulin and glucagon, are mainly induced indirectly by an organism's rhythmic feeding behavior. The function of the daily adiponectin and leptin rhythms in the daily glucose metabolism is currently unclear. An important role for the leptin rhythm seems unlikely since these rhythms are similar in rats and humans, whereas their glucose rhythms are each other's opposite.

Instead, we suggest that the SCN projections to the ANS mainly control the daily variation in glucose metabolism. We suggest that the SCN uses the ANS to prepare the body for the daily recurring changes in energy expenditure. Especially the sympathetic branch of the ANS, which innervates the liver, is important for glucose production. Its activity varies throughout the L/D cycle because the GABA and glutamate input from the SCN to the PVN oscillates\textsuperscript{198}. Peripheral glucose uptake is also modulated by the SCN, and possibly mediated via the ANS, although we were unable to show a daily rhythm in basal glucose disposal. Currently, it is unclear whether the SCN also modulates the parasympathetic input to the liver, i.e. to modulate glucose disposal or glycogen synthesis.

\textbf{Fasting}

Fasting does not disturb homeostasis acutely; because the available amount of energy sources decreases gradually, the body has some time to adapt its physiology in order
to increase the efficiency of energy expenditure. This may take several hours, as compared to the seconds or minutes that are involved in acute responses, e.g. during a hypoglycemic event. The gradual nature of the adaptation to fasting is illustrated by our data, showing that glucagon concentrations did not increase in spite of the decreased plasma glucose concentrations (chapter 2). Instead, plasma glucagon concentrations decreased during the light period. This will be discussed below.

When hypoglycemia is induced rapidly by bolus injections of insulin or 2DG (chapter 3)\(^{140,412,413}\), glucagon concentrations do rise rapidly, in order to stimulate hepatic glucose production. We suggest that the speed of changes in the glucose concentration, rather than the absolute glucose concentration itself, determines whether a counterregulatory response is induced. A rapid decrease of energy supply is potentially more life threatening than a gradual one, as the latter provides time for adaptation to the new situation. This is illustrated by glucose production by the liver. Glycogen stores in the rat are mostly depleted after 24h of fasting\(^{414}\) and a gradually increasing gluconeogenesis rate accounts for glucose production when an organism does not eat\(^{415}\).

Glucose always needs to be available to the brain, whereas other tissues may switch to energy sources other than glucose, like ketone bodies and FFAs. Therefore, glucose in the body must be redistributed to the brain\(^75\). This is accomplished, at least in part, by decreasing insulin concentrations, which prevents glucose transport into insulin-sensitive tissues and keeps glucose available to the brain instead.

Besides this redistribution of glucose in the body, energy expenditure in general needs to be redistributed, in order to save energy as much as possible. The SCN is very important in this process, as it redistributes energy expenditure in time, meaning that it determines at which time of the day energy expenditure should be allowed or prevented. Multiple studies indeed support such a role for the SCN. Body temperature, for example, is directly related to energy expenditure. In fasted rats, body temperature decreases during the resting period, but remains similar to ad libitum fed rats at the onset of the activity period\(^{16,32,244,416}\). In this way, the organism saves energy during the resting period by decreasing activity and energy expenditure, but can still be active and have the ability to search for food at the onset of the activity period. In general, the daily rhythms of fasted animals persist, but their amplitude increases because daytime levels are lower whereas nighttime levels remain unchanged.

We also report such an increase in amplitude for glucagon in fasted rats. Plasma glucagon concentrations in fasted rats were low during the light period, but did not differ from those in ad libitum fed rats at the onset of the activity period. The duration of fasting (18 or 30 hours) and the moment that fasting started (onset of activity or inactivity period) did not make a difference to this pattern. Glucagon is a catabolic hormone. Besides promoting glucose production, glucagon enhances metabolic
rate\textsuperscript{245,246,417-420}, increases thermogenesis\textsuperscript{421} and sympathetic activity to BAT\textsuperscript{422}. Therefore, decreasing its presence saves energy.

An increase in amplitude was also seen when FFAs were measured in fasted rats (chapter 4). Fasting increases lipolysis, to increase the availability of FFAs, that serve as a fuel for many tissues. During the inactivity period, lipolysis (as reflected by the plasma FFA concentration) was stimulated less than during the activity period.

Fasting has also been reported to increase the amplitude of the daily plasma leptin rhythm in fasted horses\textsuperscript{423}. Peak levels occur during the dark period and are unaffected by fasting, whereas trough levels during the light period are clearly decreased. Although this pattern in itself resembles the patterns of glucagon and body temperature in fasted rats, these leptin levels were measured in horses, i.e. in diurnal instead of nocturnal animals. Although many studies have suggested that leptin stimulates energy expenditure, both nocturnal and diurnal animals show the same daily rhythm in plasma leptin concentrations. Furthermore, leptin effects on glucose metabolism tested \textit{in vivo} are not the same as when tested \textit{in vitro}\textsuperscript{407-410}. This suggests that the central nervous system mediates at least part of the leptin effects. However, the function of a daily plasma leptin rhythm is unknown, and we propose that a simple and direct effect of (a daily rhythm in) plasma leptin on energy metabolism in general is not to be expected.

\textit{Proposed mechanism}

The MPO is involved in the regulation of body temperature via its projections to brown adipose tissue (BAT), where fuel oxidation leads to thermogenesis by means of uncoupling protein (UCP)\textsuperscript{424,425}. It is probably also involved in the mobilization of lipid stores\textsuperscript{103,343-346}. Lesions of the MPO cause an increased amplitude in the diurnal body temperature rhythm\textsuperscript{424}, even more so than in the fasted rat\textsuperscript{42}. For that reason, we suggest that the MPO may be involved in the increases in rhythm amplitude that are measured in fasting animals. Possibly, the SCN to MPO output\textsuperscript{12}, or an SCN-mediated effect on the PVN to MPO projection changes during fasting. CRF injection into the preoptic area increases thermogenesis and sympathetic nerve activity\textsuperscript{426} and it has been shown that ICV AVP inhibits CRF-induced thermogenesis\textsuperscript{427}. Reduction of thermogenesis during fasting may be mediated via the release of AVP in the MPO, which reduces corticotrophin releasing factor (CRF) release. Indeed, a change in AVP release from the SCN has been measured in food-restricted rats. A two-hour restricted feeding period at the onset of the light period led to decreased AVP content in SCN neurons\textsuperscript{428} and a two-hour feeding period in the middle of the light period was reported to shift AVP release in the SCN\textsuperscript{429}. The effect of CRF partly seems to depend on a protein called CRF-binding protein (CRF-BP), that binds and possibly inactivates CRF\textsuperscript{430}. In fasted lean and obese Zucker rats, CRF-BP expression increases
in the MPO\textsuperscript{131}. Insulin also decreases SCN activity\textsuperscript{432,433}. Therefore, fasting, and the concomitant decrease in insulin levels might cause an increased release of AVP from the SCN terminals that suppress energy expenditure. \textit{Fos} expression in the SCN of fasted rats is decreased during the light period and increased during the dark period, i.e. its daily rhythm is flattened\textsuperscript{134}. Thus, it seems that fasting indeed changes the endogenous SCN output. However, clock gene expression of fasted rats has thus far only been analyzed in peripheral tissues\textsuperscript{135,436}.

Pancreatic glucagon secretion is stimulated via the lateral hypothalamus (LHA)\textsuperscript{23,155} and the PVN\textsuperscript{198}. Indeed, retrograde transneuronal virus tracing from the pancreas revealed the most pronounced virus labeling in these nuclei. In addition, also neurons in the MPO were found\textsuperscript{92}. Possibly, the increased amplitude of the plasma glucagon rhythm in fasted rats may be mediated via a similar pathway as described above, i.e. via SCN to MPO projections. Some studies have suggested that MPO to LHA projections exist\textsuperscript{437,438}. However, PRV-labeled cells in the SCN after pancreas tracing only project to cells in the zona incerta (ZI), PVN and DMH\textsuperscript{92}. These projections contain AVP and vasoactive intestinal peptide (VIP). Possibly, MPO connections with these areas may\textsuperscript{439} modify the amplitude of the plasma glucagon rhythm.

In conclusion, the rate of energy expenditure depends on the amount of energy sources available. The primary energy source is food, so in case of plenty, metabolic rate is determined by food intake. Indeed, high fat feeding increases metabolic rate, and increased oxygen consumption has also been measured in obese people. When food is absent, the SCN determines when energy should be used, i.e. only at times when absolutely necessary. Rhythmic properties of metabolic parameters are much more pronounced in fasted animals, because they are not ‘masked’ by the effects of feeding.

\subsection*{Acute disturbances of homeostasis}

\textit{Hypoglycemia}

Insulin-induced hypoglycemia is a metabolic stressor that does not usually occur in healthy human beings. Still, it is a very relevant and often used experimental condition, as patients suffering from diabetes mellitus (DM) may experience hypoglycemic events because of their insulin therapy. Because glucose is such an important fuel source, the body senses glucose availability at multiple sites. Several hypothalamic nuclei are essential in the maintenance of glucose homeostasis and the monitoring of available glucose in particular. Sanders and Ritter concluded that counterregulatory responses are not induced by low plasma glucose but merely by a lack of (intracellular) glucose metabolism\textsuperscript{440}. This is confirmed by studies that identify the intracellular...
lular components of the glucose metabolism cascade, such as the glucose transporter GLUT2, GLUT4, glucokinase, the hexosamine biosynthesis pathway (HBP) and glucokinase to be involved in glucose or energy sensing. Activation of these mechanisms by glucose ultimately leads to an increase in intracellular ATP levels, which changes the ADP to ATP ratio. This ratio is linked to ATP-sensitive K+ channels, that change excitability of the neuron. This mechanism has been identified at peripheral and central locations, such as the hepatic portal vein, adipose and muscle tissue, and several areas in the hypothalamus and brain stem, but also in non-neuronal tissue like the pancreatic β-cells. Thus, at the organ level, changing glucose metabolism in a cell may induce an intracellular response that may also influence neighboring cells, like a paracrine effect. This is seen in the pancreas, where glucose application induces insulin release in vitro. Furthermore, sensory neurons in peripheral tissues sense glucose and change their firing rate in the way described above and send this information to the brainstem. Herewith, the ‘glucose signal’ is transmitted to other neurons, in order to induce a response when glucose availability is changing. The glucose signal reaching the brainstem from the periphery, or sensed by neurons in the brainstem itself, may induce responses (e.g. glucoprivic feeding) independent of higher brain areas, but integration with information from higher brain areas may adapt the physiological response to the peripheral amount of glucose to a specific circumstance, like the time-of-day.

When glucose concentrations suddenly drop, thus changing the amount of intracellular ATP in glucose-sensing neurons, a set of responses is induced depending on the severity of the hypoglycemia. The responses that counterregulate hypoglycemia vary over the day night cycle (chapter 3), as a hypoglycemic event may at certain times be dangerous more than at others. Especially at the onset of the activity period the counterregulatory glucagon responses are substantial. This is easily explained, as glycogen stores (both muscular and hepatic) are minimal after the resting period, during which no feeding took place. Furthermore, this is the time when the activity period starts, which increases the need for energy. Therefore, it is important at this moment to restore plasma glucose concentration to the normal range as quickly as possible. At other moments of the day, the animal can save energy by staying quiet and get away with a lower counterregulatory response, as explained above in the section about fasting.

Counterregulatory responses to hypoglycemic events are mediated via multiple redundant (i.e. serving as a back-up system for use in the event of failure of one the other mechanisms) pathways, which makes sense, given the importance of securing the availability of glucose for the brain. Furthermore, the variety of responses that occur, depending on the severity of hypoglycemia, are not likely to be mediated via just one pathway. Hypoglycemia-induced feeding behavior (glucoprivic feeding) is an
effect largely mediated via the brainstem. Decerebration or immunotoxic lesions of adrenergic cells in the PVN do not prevent glucoprivic feeding\textsuperscript{305,449}.

One of the hypothalamic nuclei involved is the PVN\textsuperscript{281}. Injection of 2DG into the PVN induces hyperglycemia\textsuperscript{305}. However, inactivation of the PVN does not prevent the counterregulatory glucagon response to insulin-induced hypoglycemia\textsuperscript{82}, supporting the notion that different aspects of counterregulation are regulated via different pathways. Inactivation of the DMH selectively attenuates the corticosterone and ACTH responses to hypoglycemia\textsuperscript{84}. Both LHA and VMH are also involved in hypoglycemia counterregulation, via noradrenergic and GABA-ergic pathways\textsuperscript{156,316,368,450}. Lesions of the VMH, glucose infusion into the VMH or inactivation of K\textsuperscript{+} channels involved in glucose sensing suppress counterregulatory responses to hypoglycemia\textsuperscript{73,288,451}.

The MPO also has glucosensitive neurons\textsuperscript{362,452} and sends projections to the LHA\textsuperscript{437}. Although there is no evidence for direct SCN projections to the LHA or the VMH, the time-of-day signal may be relayed to these nuclei via indirect connections that do receive SCN input, i.e. the (sub)PVN or MPO\textsuperscript{12}. The pathway controlling the daily rhythm in plasma corticosterone concentrations is itself mediated via direct projections from the SCN to the DMH\textsuperscript{12,19}. Possibly, this pathway also mediates the daily differences in the insulin-induced corticosterone responses that we have shown (chapter 3). This is supported by Evans and colleagues, who show that the DMH is specifically involved in the corticosterone response during insulin-induced hypoglycemia\textsuperscript{84}.

In conclusion, the SCN adapts acute responses to disturbed glucose homeostasis to the time-of-day. Furthermore, the size and duration of meals and concomitant hormone responses are modified to the time-of-day. In the hypothalamus, the SCN output is integrated with the information about high or low glucose concentrations, sensed at many peripheral and central locations.

**Meal feeding**

Although the intake of a meal is a way to maintain homeostasis rather than a disturbance, it does change the internal physiology and it elicits several types of responses that are aimed at storing the nutrients that are ingested to prevent excessive increases of for instance blood glucose levels, and at preventing excessive food intake by termination of the meal. The magnitude of the response depends on several factors, ranging from the type and amount of nutrients consumed to the time of day the meal is taken. The sight, smell and taste of the ingested food elicit quick increases in insulin and glucagon concentrations, even before any nutrients have been taken up from the gut. These very short (in rats even <3 min) responses are called cephalic phase responses and are probably mediated via the ANS\textsuperscript{453-457}. The cephalic insulin responses in the rat seem to vary over the L/D cycle. The insulin increments in the first two minutes after meal initiation are smallest at the end of the light phase\textsuperscript{230}. They are induced by factors
like taste and smell and are suggested to adapt the body's physiological response to
the nature (e.g. high or low carbohydrate content) of the ingested food. To our knowl-
edge, the function of the cephalic glucagon response has not been elucidated. We sug-
gest it may serve a purpose in preventing hypoglycemia at the time that feeding and
the concomitant insulin release have started, but no nutrients are absorbed yet.

In our rats, fed according to a six-meals-a-day schedule, we measured glucagon
responses that lasted up to 90 min after the initiation of a meal (chapter 2), possibly
mediated by amino acids in the consumed food. Total glucagon release, calculated
from the area under the curve (AUC), did not show any daily variation. However,
these data show increased glucagon concentrations at 0.5, 1.5 and 2.5 hours after the
meal, and cannot be considered cephalic phase responses.

After these quick responses, the nutrients taken up via the gut reach the circulation
and activate further nutrient sensors in the liver and pancreas, as well as several
areas in the brain. In both rats and humans, clear differences have been
shown in plasma glucose and insulin concentrations induced by meals given at differ-
ent moments during the light-dark cycle. Insulin responses are the lowest at the end
of the inactivity period (sleep), whereas glucose tolerance is the highest at that mo-
ment. This pattern is similar for multiple ways of glucose intake, i.e. iv or oral glucose tolerance tests and meal feeding, although glucose derived from
a meal induces more insulin release than iv injected glucose. Insulin sensitivity
is at its highest before the onset of the main activity period, i.e. the time when the
organism is used to eat and store the ingested nutrients. Additionally, the activity of
the organism causes a certain amount of these ingested nutrients to be oxidized im-
mediately. Therefore, less insulin is needed to induce sufficient glucose clearance, and
a more substantial insulin response would cause hypoglycemia.

Postprandial plasma lipids also display diurnal rhythmicity, and lipids are digested
more easily during the activity period. Furthermore, satiety hormones such as
cholecystokinin (CCK) and bombesin are reported to have a diurnal variation in their
ability to limit meal duration. Also the initiation of a meal depends on the pres-
ence/absence of satiety factors and on the time of the day.

Gastric emptying also displays a daily rhythm in rats and humans, with the highest
rates for emptying occurring during the activity period. A high rate of gastric
emptying may clear the way for more food, which agrees with decreased satiety dur-
ing the activity period. Different organisms are active and able to search for food only
during a specific period of the L/D cycle. Thus, multiple mechanisms allow the organ-
ism to eat and digest the most during this period.
Overstimulation leads to disease

It is possible to have too much of a good thing: this is true for many phenomena in many different aspects of life. The statement is also applicable to the regulation of different aspects of homeostasis. Although the acute response serves to reinstate the balance within the body, and is in fact a response that can save your life, the body is not built to deal with such acute responses in a high frequency. It has become clear that repeated or chronic (psychological) stress responses may lead to disease. Likewise, repeatedly occurring hypoglycemic events are dangerous as well. They impair the counterregulatory responses to subsequent hypoglycemia, a phenomenon termed hypoglycemia-associated autonomic failure (HAAF)\textsuperscript{472-474}. Even a single episode of (2DG-induced) glucoprivation may attenuate (adrenal medulla) counterregulatory and hyperglycemic responses to a subsequent glucoprivic event, if performed shortly after the first one\textsuperscript{440,475}. We did not see this effect in our rats, which underwent insulin-induced hypoglycemia twice, with at least 7 days of recovery in between. Counterregulatory failure seems to be caused not by insulin or hypoglycemia per se, but rather by a lack of intracellular glucose metabolism\textsuperscript{476}. Indeed, the decrease in counterregulatory responses is at least partly due to decreased activity of the PVN\textsuperscript{476}, although the decreased glucagon response is mediated via other hypothalamic nuclei\textsuperscript{82}. HAAF is particularly dangerous during the sleep period, when a hypoglycemic event is already less easily recognized.

Another example of over-stimulation of homeostatic responses, specifically relevant in our modern time, is feeding. Food intake in itself is necessary for survival, but the increased availability of food in our Western society in combination with decreased physical activity has made it clear that excessive food intake can be very detrimental to health. Not only the amount and frequency of food intake have increased during the last 50 years, also its timing has shifted considerably. Instead of consuming the major part of our daily intake in the morning and afternoon, we eat most at the end of the day. At this time-of-day, the energy taken in will be stored instead of oxidized. Furthermore, the need for physical activity has decreased because of technological progress. Thus, we eat too much at the wrong time of the day in combination with decreased energy expenditure. Since light\textsuperscript{7}, food intake\textsuperscript{46-48} and physical activity\textsuperscript{477,478} are powerful Zeitgebers, a shift in their presence may have a great impact on physiological parameters. A flattening in the rhythm of these Zeitgebers will result in a more constant environmental input to the brain. As has been hypothesized previously\textsuperscript{479}, such a disturbance may have profound effects on metabolism and may even cause disease.

This has become clear in studies performed with shift workers, who do not necessarily eat too much, but who do eat at unusual times. The incidence of diabetes and
cardiovascular disease is increased in this group\(^{480-483}\). Furthermore, a nocturnal lifestyle (i.e. consuming the major daily amount of food in the evening hours), induces early signs of diabetes, increased fasting glycemia and insulinemia, already after 3 weeks\(^{484}\). Altered biological rhythms have also been shown in obese subjects and type II diabetics\(^{209,485-488}\) and have been associated with cardiovascular disease\(^{489-491}\). The leptin rhythm in the rat is blunted after high fat feeding and absolute levels are increased after 6-meals-a-day feeding\(^{314,492}\). This indicates that the type of food and the timing of feeding may alter circadian rhythms. On the other hand, healthy first degree relatives of diabetes patients also show decreased amplitude in daily rhythmicity\(^{493}\). Therefore, although altered biological rhythms are clearly related to disease, it is not known whether they should be considered cause or consequence.

In addition, the increased frequency of eating by itself may be detrimental. Each time we eat something, an insulin response is induced. Chronic intracranial insulin infusion results in disturbed feeding rhythms\(^{494}\) and altered SCN glucose utilization\(^{133}\) in rats. Animals receiving ICV insulin eat less during the dark period and more during the light period. Besides the effect of feeding on daily rhythms in physiology, chronic hyperinsulinemia itself appears to affect health. Women with high fasting insulin concentrations have an increased risk for breast cancer\(^{495}\).

Taken together, it seems that the circadian system that controls energy homeostasis and has evolved during a time span of thousands of years, cannot cope with the disturbances in the daily activity pattern induced by the 24-hour society that has evolved during the last 50 years.

**Conclusion**

The biological clock is in many ways important for keeping glucose homeostasis between its necessary narrow boundaries. We have described here that not only anticipatory homeostasis is organized by the SCN but that also acute responses to disturbances of homeostasis are modulated by the SCN. Because the internal balance in the body is determined by the time of the day, a disturbance will result in a response which will differ according to this balance, to fit the specific needs of that time of day. Furthermore, diurnal rhythms adapt nutrient availability and energy expenditure to fit the activity state, thereby preventing unnecessary disturbance of the delicate homeostatic balance. Daily rhythms anticipating metabolic processes become even more important when energy is restricted, i.e. during fasting. By lowering body temperature and other catabolic processes, energy is saved during the inactivity period and can thus be used very efficiently in times of need. Naturally, the disturbance of metabolic homeostasis that is most dangerous is hypoglycemia, i.e. a shortage of energy. However, hyperglycemia, in the long run is also dangerous. In diabetics with
poor glycemic control, severe damage of the vasculature may occur. Furthermore, it de-regulates normal metabolism, including biological clock control of metabolism, as reflected by the flattened metabolic rhythms. An increasing body of evidence shows that disturbed daily rhythms are indeed associated with disease, either as a cause or as a consequence. The coincidence of disturbed daily rhythms and obesity and/or diabetes indicates how important an adequate regulation of daily glucose metabolism may be.

Experiments described in this thesis indicate that modulation of daily basal glucose homeostasis by the biological clock is mainly mediated via neuronal pathways. We hypothesize that the SCN shifts the balance between sympathetic and parasympathetic output of the hypothalamus, which determines if and where glucose will be stored or mobilized. Probably, there is not just one pathway that the SCN uses to adjust glucose production or utilization; in other words, it may be impossible to single out just one mechanism that causes the daily rhythm in basal glucose concentrations. Maintaining glucose homeostasis is of such importance that it involves many redundant (i.e. both overlapping and supplementary) mechanisms, and several different hypothalamic nuclei may therefore play a role in the fine-tuning. The different pathways that are in some way involved in the SCN control of glucose homeostasis may be identified by a series of carefully designed experiments. Different experimental circumstances may lead to different interpretations. In case of acute disturbances of homeostasis, a number of counterregulatory measures may be taken, depending on the severity of the disturbance and the nature and location of the stimulus. Glucose may be sensed in all cells in the body, but when it comes to responses to aberrant glucose availability (or energy availability in general), there is a hierarchy to where glucose is sensed and what effect a certain glucose signal may have. Small local changes, i.e. at the cell level, in the presence or use of glucose may lead to local adjustments. These may be overruled, however, by a response at the organ level when this is necessary. On the top of the hierarchy is the central nervous system that normally utilizes only glucose. Information about glucose or energy availability at different locations in the body reaches the brain via sensory neural feedback and the blood stream. This information is integrated in the brainstem and hypothalamus, resulting in an appropriate output to the periphery. The central nervous system always ensures its own supply of glucose by orchestrating the distribution of glucose in the body. This redistribution is most obvious during food restriction, when the body completely depends on its own endogenous energy supply.

**Future studies**

Additional studies should be directed at unraveling the neural pathways that the SCN
uses to affect peripheral glucose homeostasis in more detail. Several hypothalamic nuclei may be involved in the stimulation or inhibition of glucose production or storage, and may therefore be part of the SCN-to-periphery pathways that modulate glucose homeostasis. In order to understand the complex regulatory system, it is necessary to lift out specific nuclei or neurotransmitters and investigate their function repeatedly under different experimental conditions. Only careful interpretation of a mixture of data obtained from different experiments can build a better view on how the complete regulatory system works.

It is already clear that the sympathetic innervation of the liver is important for glucose production. The implication of the parasympathetic input to the liver in the daily control of glucose homeostasis, however, is not clear yet. We hypothesize that parasympathetic innervation is important for the control of glucose storage in different organs. Specific denervation studies, in combination with activating or silencing vagal output should help to reveal this. Silencing specific nuclei or neurotransmitters may be done by means of the microdialysis technique.

Furthermore, the rhythmic properties of glucose production, by the liver but possibly also the kidneys and intestines, have not been elucidated. A daily rhythm in hepatic glucose production may be revealed by the use of clamp studies. Ideally, we would like to investigate metabolism in the basal state, i.e. without evoking unnatural responses or putting the body in a constantly unnatural, i.e. hyperglycemic, state. At this moment, however, using the clamp technique may be the only way to take a step in the direction of identifying the mechanism by which the SCN controls glucose production. The same holds for peripheral glucose uptake. Although clamp studies cannot be used to measure basal glucose uptake, they may be useful to identify differences between peripheral organs. Purely basal glucose uptake may be estimated, however, by the analysis of expression and activity of e.g. glucose transporters, hexokinase and glycogenic enzymes. It is important to combine gene expression studies with enzyme or transporter activity, in order to interpret these data correctly. Such an approach may give more insight in how the SCN controls glucose uptake.