Effects of feeding time and light on energy metabolism in rats
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General Discussion
The predictability of the daily sunrise and sunset during evolution equipped most species, including mammals, with a complex internal clock mechanism. Metabolic adaptation to the light and dark phase of a day requires modifications in gene expression, organ activity and behaviour. Central regulation of the countless bodily daily rhythms is in the hands of the suprachiasmatic nucleus (SCN) in the brain. On top of the crossing of the optical nerves is the SCN conveniently located to perceive light information from the environment. Multiple, sometimes parallel running pathways downstream of the SCN forward time and light information to the rest of the body. These pathways sprout from several hypothalamic brain areas where signals from the SCN are integrated with signals on the homeostatic status of the body. Acute energetic messages from periphery, for instance hypo- and hyperglycaemia, may interfere with the SCN-derived outgoing signals, potentially causing conflicting commands for intermediate brain areas and (subsequently) peripheral organs. In addition to these central commands, local molecular clocks in the periphery contribute to metabolic and circadian activity of organs. Disruption of these peripheral clocks has been shown to decrease metabolic health [1–7], supporting the tight integration of the circadian system and energy metabolism.

An increasing amount of work and leisure activities in human life takes place in the evening and beginning of the night, an unnatural time for diurnal species, including humans, to be awake, ingest food and see light. Shift workers, and in particular night workers, frequently disrupt their regular day and night rhythm, usually for several consecutive days during many years. The increased risk of shift workers to develop metabolic diseases [8–21] is considered to originate from the exposure to severe circadian disruption. Several factors may be, partially, responsible for the increased risk of metabolic disease in shift workers, including exposure to unusual timing of activity and sleep, feeding and fasting, (natural) light and darkness and social interactions. The underlying mechanism of how these shift work conditions relate to the pathophysiological disruption of metabolism is increasingly investigated, but remains so far unknown. The aim of this thesis was to study in more detail the metabolic consequences of two shift work conditions; feeding and light exposure at an unusual time-of-day, and thereby to expand the understanding of the mechanism behind the potentially disruptive effects of these conditions on energy metabolism. In the first part of this
thesis, chapters 3 and 4 describe the effects of abnormal timing of food intake on parameters including behaviour, adiposity, endocrine control and circadian rhythmicity in peripheral organs and the brain. In the second part, chapters 5, 6 and 7 focus on the acute effects of circadian disruption by exposure to light at night and the consequences for glucose metabolism and the liver transcriptome. All experimental studies in this thesis were carried out with male Wistar rats.

In Chapter 2, we reviewed the range of animals and experimental setups that are used to model human shift work conditions, with a focus on the consequences for metabolic health. During the past decade, the number of studies claiming to use a shift work model has increased largely, together aiming to improve the medical condition of millions of human individuals around the world. Indeed, models using unusual timing of light exposure by shorter or longer photoperiods or constant (bright or dim) light conditions were greatly disruptive for body weight and glucose metabolism. Restriction of the timing of food intake was the most frequently used experimental setup and mainly disrupted glucose and lipid metabolism, but less frequently also affected the amount of food intake and locomotor activity. Models using primarily a disruption of activity and/or sleep were lower in numbers. Studies for activity were limited and only performed with rats, whereas sleep models presented a variety of methods (e.g. sleep-deprivation, -restriction, -disruption) and mainly disrupted glucose metabolism. Inconsistency in experimental design was not only found in the sleep studies, but, for instance, also the duration of the aberrant light exposure or feeding conditions ranged from a few days to months. Furthermore, the number of studied parameters was sometimes limited, in particular sleep measurements were minimal. Clearly, all models affected any of the metabolic parameters, but a larger number of studies with greater methodological consistency will be needed before firm conclusions can be drawn.

Furthermore, in Chapter 2, we discussed and expanded the hypothesis on how these shift work models lead to adverse metabolic effects through circadian disruption. We distinguished four bodily levels of circadian disruption, as a consequence of the desynchronization between an organism and its environment (Fig. 1). The distinction of the different levels provides a more systematic method to get insight of metabolic consequences by circadian disruption. The highest level we distinguished was a desynchronization between the central clock and
the peripheral clocks, occurring when clock genes in peripheral organs adapt to, for instance, food intake during the sleep period, but rhythms in the SCN stay put to the L/D-cycle [22]. Level 2 comprises desynchronization between separate organs, for instance when the liver and muscle respond differently to feeding at the wrong time-of-day [23]. At this level, we also included desynchronization between non-SCN brain areas, which may occur in (hypothalamic) nuclei integrating SCN-gated signals with afferent peripheral signals [24]. Desynchronization at levels 3 and 4 occurs intracellularly between clock genes and clock-controlled genes (CCGs; level 3) or between the molecular clock genes themselves (level 4) [25–29]. These types of desynchronization may take place in each organ or cell when synchronizing signals for cellular activity are incongruent. In the level 2 desynchronization, we also included a possible desynchronization between anatomical distinct parts of the SCN, occurring, for example, due to a discrepancy between the responses of the ventral and dorsal SCN to a phase shift [30, 31]. However, in hindsight, this may also hold true for other hypothalamic nuclei and peripheral organs, containing separate anatomical sections or multiple cell types. For instance, the paraventricular nucleus (PVN) of the brain contains many different cell types, such as pre-autonomic neurons, parvocellular neuroendocrine neurons, oxytocin- and vasopressin-secreting magnocellular neurons, and interneurons. These cell types are clustered to some extent in distinct anatomical subzones of the PVN. Moreover, cellular heterogeneity is also found in important metabolic organs such as the liver (with different lobules and hepatocytes, Kupffer cells and stellate cells), in muscle tissue (with aerobic and anaerobic muscle fibres) and in the pancreas (with alpha- and beta-cells). These levels of desynchronization, as well as levels 3 and 4 have only been marginally studied and should be investigated in the future as they may also play an important role in organ dysfunctionality.

**Part I (Chapters 3 and 4)** of this thesis focused on food intake, an important Zeitgeber for many peripheral clocks, which may act as a driving oscillator for several circadian traits that ignore the L/D-schedule [32, 33]. The worldwide obesity pandemic raised a strong interest in the importance of many aspects of food intake for health [34], the close interactions between food intake and the circadian system being one of these aspects [35]. Experimental designs to study the effects of feeding
time are diverse, but most of them involve a shift in the timing of food intake, sometimes combined with a restriction of the amount of calories. An early study with mice demonstrated that high-fat feeding during the light phase increased body weight [36], demonstrating an interaction between feeding time and health. Numerous studies followed (a selection [23, 37–50]), aiming to narrow down the time-window for negative health consequences, to test possible protective or reversing mechanisms, and to investigate contributing mechanisms. Although experimental variation is large between these studies (chow or high-fat diet, length of experiment, included parameters, rodent species), generally each study demonstrated some effects on metabolic health. Our data are congruent with these studies, observing increased adiposity, decreased food intake and shifts in the rhythm of locomotor activity and the respiratory exchange ratio (RER). In addition, the mechanism of how mistimed food intake affects metabolism was further investigated by several studies, including ours.

First, the rhythm of voluntary food intake is strongly intertwined with the rhythm in locomotor activity. We, and others, found that restricting food availability to a non-physiological phase of the day causes a shift in locomotor activity, a first sign for circadian disruption that cannot be excluded as a major factor in the development of metabolic effects. However, the rhythm of activity did not fully synchronize to feeding time, suggesting that locomotor activity, as an important output of the SCN, remains partly entrained to the L/D-cycle, even after 8 weeks of timed food restriction. Some studies subjected rats to a working protocol (i.e., forced minimal activity during the light phase) and provided food during the night or day, aiming to discriminate between food and activity as the leading pathological factor. These studies showed that despite the anti-phasic availability of food with the forced work activity, food during the natural time-of-day led to lower body weight, reduced adiposity and maintenance of rhythmicity in hormone concentrations when compared to day-working animals eating in synch with work, but out of synch with the SCN [24, 28, 39]. Thus, feeding time seems to be dominant over timing of locomotor activity in causing negative metabolic consequences.

The partly shift of locomotor activity demonstrates the first level of desynchronization identified in Chapter 2. In Chapters 3 and 4, we also provided examples of desynchronization levels 2 to 4 by studying
rhythms of clock and metabolic gene expression in multiple brain areas and peripheral organs. Although the hepatic clock is relatively easy to study (with regard to RNA isolation and obtaining strong oscillations), the liver is not the sole organ responsible for the control of energy homeostasis. For instance, glucose metabolism depends on direct and well-regulated interactions between the liver, pancreas, gut and muscle tissue. Therefore, it is essential to investigate whether responses to an aberrant timing of food intake in these peripheral tissues run in parallel or apart. In line with previous studies [22, 23, 51], liver rhythms adapted to the new feeding schedule by shifting all genes investigated. Interestingly, the rhythmic gene expression observed in muscle tissue of *ad libitum* and dark fed animals disappeared in animals fed during the light phase. Except for rhythmic, but shifted, *Uncoupling protein 3* (Ucp3) expression, all genes became a-rhythmic, potentially displaying reflection of the reduced amplitude in the daily activity rhythm. Others also observed effects of feeding time on gene expression rhythms in the muscle and white adipose tissue [52, 53]. Thus, food-induced circadian disruption hits the body in more places than the liver. This change in feeding time completely shifts the rhythms in the liver, and leaves the muscle a-rhythmic: desynchronization at level 2 (Fig. 1). Imaginably, these unequal shifts in local molecular clocks may lead to miscommunication between these organs, which is a clear risk factor for a derangement of the control of glucose and lipid metabolism [54, 55].

Our study did not provide major indications for intra-organ or intracellular desynchronization (levels 3 and 4, Fig. 1), as we did not find large differences between genes of the molecular clock or between clock genes and CCGs. This consistency between gain, loss or phase shifts of rhythms in both clock and metabolic genes is congruent with the mechanism of entrainment by food. Postprandial absorption and oxidation of nutrients in order to produce adenosine triphosphate (ATP), generates intracellular modifications in signalling molecules, which influences the expression of clock genes. For instance, changes in the ratio of nicotinamide adenine dinucleotide (NAD) and its phosphorylated form (NADH) affect CLOCK/BMAL1 stability, but also peroxisome proliferator-activated receptor (Ppar) gamma coactivator 1-alpha (Pgc1α) and other members of PPAR family are nutrient-responsive and directly interact with the molecular clock [55–58], besides their function as metabolic regulatory transcription factors [59]. Indeed, these
genes (Ppara, Pgc1α, Nicotinamide phosphoribosyl transferase; Nampt) were affected similarly to clock genes in our study, further supporting this pathway of hepatic entrainment by food. However, this contradicts another study describing uncoupling of Ppar and Nampt from clock gene rhythms in liver by daytime feeding [28]. Altogether, food entrainment occurs locally at the level of the liver through direct nutrient sensing on hepatocytes affecting the intertwined web of metabolic and clock genes. How signals from hormonal (e.g., glucose, glucocorticoids, insulin, fibroblast growth factors) or neural pathways (by the autonomic nervous system) are integrated in this process at the level of cells, remains to be studied. Furthermore, it remains unknown whether activation of intracellular signalling is similar in different organs. For instance, skeletal muscle is an important metabolic organ, but it is, as yet, unknown how entrainment by food and by exercise [60, 61] are integrated in muscle tissue and whether this affects metabolic functions such as insulin sensitivity and glucose uptake [2, 54].

**Future directions:** Since the molecular clock is present everywhere in the body, it is important to include other metabolically important organs, such as the intestines [62], pancreas and brown and white adipose tissues, in further studies to investigate their sensitivity to food entrainment. Expansion of the descriptive studies including other organs will be helpful in providing a more complete overview on the consequences of food timing, altogether important to study rescuing or preventive methods at a later stage. Before doing so, data are needed on desynchronization between other organs than SCN, liver and muscle, but also possible intra-organ desynchronization ranging from desynchrony between anatomical distinct parts, as well as, between different gene groups (i.e., metabolic versus clock). In addition, many studies, including ours, report mRNA expression without data on protein content or enzymatic activity. Nevertheless, these data will be necessary in order to understand better the functional significance of phase shifts, losses or gains of rhythmicity in gene expression.

The hypothalamus of the brain, the major control centre for energy homeostasis, certainly registers a shift in the timing of food intake, since it directly responds to feeding related changes in glucose, insulin and leptin. The SCN has been demonstrated to be relatively resistant to changes in feeding time, but little is known about how other hypothalamic nuclei respond to feeding at an unusual time-of-day. We
hypothesized that afferent (hormonal) signals from the periphery will clash with SCN-derived time signals, disrupting the normal activity rhythms of hypothalamic nuclei. In particular, we targeted the orexin system in our experiments. Orexin, also known as hypocretin, is a neuropeptide solely produced in the lateral hypothalamus (LH) and identified as a key player in sleep/wake regulation [63, 64]. Evidence increases on the role of orexin in metabolic and circadian functions. On the one hand, interactions with the circadian system exist: projections from and to the SCN were demonstrated [65–67], and daily fluctuations were found for peptide content in the cerebrospinal fluid (CSF) [66, 68, 69], neuronal activity [70, 71] and, to a lesser extent, mRNA in brain tissue [72, 73]. On the other hand, orexin neurons respond acutely to changes in glucose, leptin, and ghrelin concentrations [74]. On their turn, orexin neurons directly control glucose [75, 76] and lipid metabolism.
by stimulation of glucose production and uptake [77], as well as food intake [78–80], amongst other functions [81, 82]. We hypothesized that the orexin system would have a central role in the metabolic problems resulting from circadian disruption. Hypothetically, the regular (rhythmic) activity of orexin neurons will be attenuated when afferent signals from the SCN and periphery are misaligned, ultimately leading to negative metabolic consequences. Therefore, we studied mRNA expression of (prepro)orexin and its two receptors in tissue of the LH after 3 (Chapter 4) or 8 weeks (Chapter 3) of shifting the timing of food intake. Both studies described a lack of significant daily variation in Orexin (Orx) or Orx-receptor 2 in ad libitum feeding conditions, which remained unaffected by daytime feeding. The non-existence of diurnal fluctuation contradicts several studies reporting rhythmicity in the orexin system, although other studies too reported minimal or absent oscillations in mRNA [78, 83]. Nevertheless, expression of Orx-receptor 1 showed daily fluctuations that disappeared with daytime feeding, suggesting that changes did occur in the orexin system. Data are limited on the functional differences of the two receptor-types [84], and whether receptor-mRNA correlates with translation of the peptides (Orexin A and B) or their release. Altogether, we found no clear evidence to confirm the hypothesis that orexin plays a major role in the metabolic disruptions after alterations in the timing of food intake, although also no evidence was found to dispute this hypothesis.

Multiple hypothalamic neuropeptides, besides orexin, are engaged in the control of energy metabolism. Therefore, Chapter 4 also includes data on the expression of Melanin-concentrating hormone (Mch) and Neuropeptide Y (Npy) in brain punches of the LH and the arcuate nucleus (ARC), respectively. Both neuropeptides have been extensively reported to participate in food intake and to interact with the SCN [85–87]. However, daytime feeding did not significantly affect expression of Npy and Mch in our study. Again, daily variation in the expression of these genes was lacking under ad libitum feeding conditions, complicating the detection of effects of a shift in feeding time. Interestingly, the core clock genes Period 1 (Per1), and Per2 demonstrated clear daily fluctuation in both the LH and ARC, but these were disrupted by daytime feeding. This indicates level two desynchronization, i.e., mismatch between SCN and non-SCN brain areas, and perhaps also level three of desynchronization: metabolic genes (i.e., the neuropeptides)
and the core clock within the same area are differentially affected. It is unknown what the consequences are of a loss of rhythmicity in either the LH or ARC, two major target areas for communication with the SCN and with each other. It is important to note that a punch from the ARC or LH includes several cell types. Therefore, it is unknown whether the observed losses in molecular clock gene rhythms occurred in all cell types or in a particular subset of cells. In conclusion, the studies of Chapters 3 and 4 demonstrate that shifting the timing of food intake indeed affects the molecular clock in individual hypothalamic nuclei. A first suggestion was found for modulation of the orexin system, but this has to be investigated further, as well as for other neuropeptides.

**Future directions:** Chapters 3 and 4 consistently present small or absent effects on expression of neuropeptides and clock genes in the LH. The cellular heterogeneity in the LH and other hypothalamic nuclei is large, therefore future studies should further study effects of circadian disruption by food on individual cell types. Data on tissue and CSF content or secretion levels of the orexin peptides would better test the hypothesis whether the orexin system is involved in the development of adverse metabolic effects after circadian disruption. The use of knock-out mice for orexin or its receptors, to study the role of orexin, is less preferable since these animals develop narcolepsy and thereby firstly suffer a sleep disorder, which is in itself accompanied by health problems [80]. Furthermore, consistency in effects on brain, liver, muscle and whole body metabolism (i.e., body weight, adiposity, RER rhythm) between chapters 3 and 4 shows that 3 or 8 weeks of exposure to daytime feeding does not change results much. Many studies, including ours, expose animals to a ‘constant’ shift work condition. This is accurate when focusing on acute effects of the condition, but does not mimic human shift work conditions. Since shift workers alternate between disruptive conditions (i.e., working at night) and healthy conditions (i.e., working during the day), it is important to investigate whether the shifts in liver rhythms or loss of muscle rhythms are restored and recovered in the non-disruptive period. Recent studies have investigated the potential of caffeine [88], exercise [89, 90], and nutrients [91] to influence and restore normal clock rhythmicity, although such studies could be extremely helpful more studies will be needed in order for the shift working population to really profit.
Part II (Chapters 5, 6 and 7) of this thesis concentrated on more acute effects of (circadian) disruption on energy metabolism, in particular the effects of light at night on glucose homeostasis. Light radiation is converted into electrical currents after absorption by photosensitive retinal cells. By transmission through the optical nerves, light signals reach areas important for vision, the pupil reflex and circadian entrainment. Stimulation of Per1 expression in the SCN after reception of light signals [92, 93] is the daily start of entrainment of the central clock and with that the most dominant Zeitgeber for mammalian entrainment. Intrinsically photosensitive retinal ganglion cells (ipRGCs) are responsible for the retinal input to the SCN, but have also been demonstrated to send projections to other hypothalamic and thalamic areas [94, 95], hypothetically affecting other aspects of physiology. The retino-hypothalamic tract (RHT) is considered to be important for non-image forming (NIF) functions of light, which are increasing in number. Besides well-known immediate effects on melatonin secretion, now arousal, mood and metabolism too are reported to be acutely affected by light exposure. In Chapters 5 and 6, we added glucose tolerance as a metabolic function acutely affected by light signalling. The initiation and design of this study arose from previous experiments in our lab, demonstrating that glucose homeostasis is strongly under the control of the SCN, including rhythms in plasma glucose levels and glucose tolerance [96–98]. Moreover, light exposure at night (LAN) was found to (SCN-dependently) affect peripheral tissues [99–101], including the liver, which is an essential organ in glucose metabolism. In particular, exposure to 1.5h LAN stimulated mRNA expression of phosphoenolpyruvate carboxykinase (PEPCK), an enzyme directly involved in hepatic glucose production [99]. Our experiments demonstrated that exposure to LAN indeed acutely affected glucose homeostasis as tested by intravenous glucose tolerance tests. Two hours of LAN reduced glucose tolerance as reflected by increasing glucose concentrations in the beginning of the dark phase (at Zeitgeber Time (ZT)15), and increasing insulin levels at the end of the dark phase (at ZT21). The effects on glucose tolerance, at ZT15, depended on intensity, duration and wavelength of the LAN exposure, but were not affected by fasting or a high-fat diet.

The discrepancy between the LAN-induced effects at ZT15 and ZT21 indicate that the circadian clock might play a role in the effects of LAN on glucose tolerance. It remains unknown whether the discrepancy
arises at the level of the SCN, perhaps through selective gating of light signals, or locally in the peripheral clocks that are affected by LAN [99–101]. The hyperinsulinemia at ZT21 reduced glucose levels to control values, suggesting that LAN decreased glucose uptake at both ZT15 and ZT21. Most likely, the LAN-induced reduced glucose uptake at ZT15 resulted in hyperglycaemia due to a simultaneous malfunctioning of the glucose-sensing pancreatic β-cells to increase insulin release. Thus, apparently, LAN affects different tissues simultaneously. The main suspect to facilitate this mechanism is the autonomic nervous system (ANS), which is under strong control by the SCN and PVN in the hypothalamus [102, 103]. Indeed, the nerves of the ANS were described to acutely change their tonus after light stimulation [104, 105] and to be involved in light-effects on peripheral gene expression [99, 100]. LAN exposure stimulated the sympathetic hepatic efferents, and this increased sympathetic activity may lead to a diversity of actions. Firstly, hepatic glucose production increases [106], although it is questionable to what extent this occurs after an infusion of exogenous glucose and we found no effects after one hour of LAN on baseline glucose levels. Furthermore, sympathetic stimulation reduces insulin secretion, contributing to reduced glucose uptake by insulin-sensitive tissues [106, 107]. Potential effects of light on secreting activity by the pancreas are in line with the demonstrated reduced vagal pancreatic activity by light [104]. Interestingly, sympathetic hepatic stimulation likely reduces glucose uptake by the liver. It was found that, after a peripheral glucose infusion, hepatic glucose uptake was stimulated, which raised further after a sympathectomy of the liver [108]. Though results are less pronounced, it was suggested that hepatic sympato-excitation decreases glucose uptake by muscles [108, 109]. Therefore, perhaps, if LAN exposure stimulates sympathetic activity to the liver and thereby interferes with hepatic (and non-hepatic) glucose uptake, this may result in hyperglycaemia, especially when insulin release is insufficient (Fig. 2).

**Future directions:** Experiments should focus on testing glucose uptake mechanisms, both hepatic and non-hepatic, to examine in more detail the underlying pathway of LAN-induced glucose intolerance. Additionally, it is important to study pancreatic function, as hyperglycaemia at ZT15 may have resulted from a combination of decreased glucose uptake and failing β-cellular activity. Interestingly, the lack of insulin response was only found at ZT15, and not at ZT21, pointing towards a
role for the SCN. SCN lesion or silencing studies would provide more information on the involvement of the master clock, perhaps either by time-dependent gating of the light signal or stimulation of the autonomic nerves. Finally, all experiments were performed in a naïve acute setting, and it remains to be studied whether the effects on glucose tolerance are repetitive or long lasting when tested in different setups.

**Chapters 5 and 6** demonstrated that the induction of glucose intolerance by LAN depends on the wavelength, intensity and duration of the light exposure. Particularly in the circadian field, interest has raised recently in the importance of light-wavelengths for physiology. The discovery of melanopsin [110] as the functional photopigment in ipRGCs [111] stimulated the field of circadian photoreception, by showing its importance for the regulation of numerous NIF functions of light. Essential circadian functions including suppression of pineal melatonin secretion by light [112, 113] and light entrainment of behaviour [114, 115] were found to be regulated by ipRGCs, which are most sensitive to exposure of light with short wavelength [111, 116–119]. Spectral sensitivity functions, in human studies too, demonstrated largest potential for blue light (i.e., short wavelength) to modulate alertness, melatonin and cortisol levels, amongst other functions [113, 120–124]. Rods and cones are the two major retinal photosensitive receptors, important for vision, but their effects are also mediated via ipRGCs [125]. In Chapter 5, we used green (peak irradiance at \( \lambda \) 521nm) and blue (\( \lambda \) 460nm) LEDs with isoquantal log photon flux (14.11) to test the spectral sensitivity of the glucose responses to light. Interestingly, we found effects of LAN exposure to green, but not blue light. These results provide a first indication for the mediating retinal pathway, as maximal absorbance of middle-wavelength cones (M-cones) lies within the green spectrum. However, melanopsin-containing photoreceptors also respond to the green spectrum despite their optimal sensitivity within the blue spectrum. Our data are in line with a recent study describing distinct responses of sleep and arousal functions to green over blue light exposure, showing activation of separate brain areas (green induces cFOS upregulation in ventrolateral preoptic nucleus (VLPO), blue in SCN), and demonstrating a role for a subtype of melanopsin-containing cells in green light transduction [126]. The VLPO is a well-known brain area to regulate sleep/wake behaviour, through projections to orexin neurons, amongst others [127]. Orexin neuronal activation has
been described after light exposure [128] and to mediate glucose homeostasis through the autonomic nervous system [76, 77]. These datasets together feed speculation on a role for the orexin system in the LAN-induced effects on glucose tolerance (Fig. 2). However, whether the disruption of glucose homeostasis by exposure to LAN depends on melanopsin or cone photoreceptors, involves the orexin system, acts through the autonomic nervous system and shows equal responses in human individuals [129–131], all remain to be studied.

Chapter 7 describes our first trial to demonstrate the role of the autonomic nervous system (ANS) in transmitting light signalling to the liver. The data on glucose tolerance in Chapters 5 and 6 suggested the liver to be a major target to receive acute light signals, in line with the previously observed effects on gene expression [99]. We expanded these observations by using a microarray approach to study the complete liver rat transcriptome after light exposure. Despite the relatively small changes (i.e., only slight fold changes), LAN exposure distinguishably modified the transcriptome and mainly targeted hormonal signalling pathways. This fits with the hypothesis that the signals of light run through the hypothalamus, including the PVN and potentially modulate the activity of (endocrine and pre-autonomic) signalling neurons. Removal of the hepatic sympathetic and parasympathetic nerves modified the response of LAN on the liver transcriptome, but did not prevent all LAN-induced up- and downregulations. Thus, the ANS indeed facilitates a pathway for light signals to affect the liver transcriptome acutely (Fig. 2), but to what extent this is mainly the sympathetic or parasympathetic nerve, is left to study. In addition, it is clear that a major part of the light effects reaches the liver through alternate pathways. Potentially other neuroendocrine pathways (e.g., HPA-, HPT-, HPG-axes) or neuro-autonomic pathways (e.g., direct innervation of adrenal glands, pancreas, or others) are affected by light, which peripherally act on the liver.

The importance of a functional ANS has been described frequently, as an imbalance of the ANS is associated with metabolic disorders, including diabetes mellitus and obesity [132]. Moreover, the role of the autonomic nerves in glucose metabolism has been well established, including the hypothalamic control of autonomic activity by pre-autonomic neurons from the PVN in relation with the SCN. The ANS is a potential pathway for the SCN to orchestrate synchronization between peripheral organs and the master clock itself through fast and direct connections,
in order to, for instance, optimally control liver participation in glucose control (Fig. 2). However, it is rather unclear how the ANS acts locally in the liver to impose its signals on to liver function and modulate metabolic activity. Through a transcriptomics approach combined with selective denervations, we aimed to identify new target genes or pathways at which the autonomic nerves interact with the liver. Removal of one or both hepatic nerves facilitated small but consistent changes in gene groups, with the fewest changes induced by a sympathectomy. Removal of the parasympathetic nerve induced more modifications, which were most similar to the total denervated group. Studying the effects in more detail revealed a few interesting pathways affected by the denervations. Indeed, metabolic pathways, in particular at the level of intracellular signalling were affected by the denervations. Moreover, the data suggest that the ANS directly acts on the molecular clock in the liver, as the Circadian rhythm pathway was affected by both a parasympathectomy and total denervation. Theoretically, using the ANS to shift/modulate the molecular clock in the liver, and potentially other peripheral tissues, would be a highly efficient way for the SCN to synchronize the complete body to a new rhythm. Indeed, several studies have tried to demonstrate the importance of the SCN-ANS axis for peripheral synchronization by showing that SCN lesion affects peripheral rhythmic expression [133, 134], but ANS denervation of individual organs modulated but did not completely abolish peripheral rhythms [25, 133–136].

**Future directions:** It was shown that peripheral entrainment acts through ipRGCs and through the non-molecular light-stimulated function of the SCN [137–139]. We now added to this that the ANS partly facilitates light signalling to the liver, potentially by acting on the local molecular clock. Future studies by using phase-shifting paradigms with autonomically denervated animals, should investigate whether peripheral entrainment is indeed facilitated by the autonomic innervation of peripheral organs. Thus far, the interaction between the ANS and clock entrainment was only shown by a sympathetic denervation of the submaxillary gland leading to increased sensitivity to food-induced phase shifting [136]. Furthermore, the number of affected physiological functions by light is increasing, and our microarray study suggests involvement of other endocrine systems too. It would be helpful if more hormonal functions will be tested for their sensitivity to nocturnal light exposure, as was done only for a few until now [140–142].
Altogether, this thesis provides additional data on the broad effects of circadian disruption in shift work conditions. We used animal models to mimic human shift work conditions in both a chronic (i.e., weeks of food-restriction) and acute (i.e., single light exposure at night) setting. The use of animals is essential in order to investigate effects at more cellular and molecular levels than possible in humans. Ideally, results translate well to human physiology, but it is unclear how well animal and human data match. Nevertheless, the number of human studies is increasing and point in the same direction. The importance of the timing of food intake was found to correlate with metabolic health [143–146].

**Figure 2. Hypothetical model on the neural pathway facilitating acute effects of light on glucose metabolism.** Light signals are absorbed by melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) and cones, which directly or indirectly (via the SCN) forward the light signals to other brain areas, including the ventrolateral preoptic area (VLPO). Further signalling may go through (orexin neurons in) the lateral hypothalamus (LH), towards the paraventricular nucleus (PVN) containing pre-autonomic and endocrine neurons. Stimulated sympathetic and inhibited parasympathetic efferent nerves reach (the molecular clock of) several peripheral organs. This may lead to modifications in glucose production, glucose uptake (in liver, adipose tissue, muscle) and pancreatic insulin release. For clarity, potential other involved (endocrine) organs and pathways are not displayed in this scheme.
including the potential to lose weight [147–150]. Although the increased risk to develop metabolic diseases in shift workers manifests itself only after years, circadian misalignment paradigms in volunteers of a few days or weeks only have demonstrated to already cause circadian and metabolic disruption [16, 151–157]. To further investigate internal desynchronization, for instance between organs, is more challenging in human participants and awaits better circadian biomarkers, in addition to the widely used melatonin rhythm. Indeed, development of easily accessible biomarkers reflecting internal circadian time, ideally unique for individual organs, is an important topic of circadian research [158].

Effects of exposure to light at night has also been investigated in human subjects, mainly with reference to sleep problems, mood disorders and cancer development [159, 160]. Since nocturnal light exposure is an almost inevitable condition for people worldwide due to environmental light pollution [161, 162], this has been studied for correlations with metabolic disruption [163, 164]. Equally, the increasing time people spent indoors leads to possible effects of underexposure to daytime (sun)light; a topic that has been much less investigated in the circadian field but probably contributes to physiological problems as well [165]. Our experimental design tested the more acute efficacy of LAN, which has been studied minimally in human subjects. Moreover, focusing on the disruptive potential of light for glucose metabolism is relatively new, besides a few animal studies using chronic LAN [166–168]. During the time of our experiments, a few related human experimental studies were published describing acute effects of light exposure on glucose homeostasis. It was shown that bright light exposure in a single night increased plasma glucose and insulin [129], blue-enriched evening light also induced a higher peak in glucose [130], and bright morning light caused higher glucose in patients with diabetes type 2 [131]. Until now, however, the effects of green light on glucose control have not been investigated in humans.

Altogether, disruptive effects were found in both animal and human experiments for feeding and light exposure studies. The congruency between metabolic disruption in both animal and human studies validates the use of animal models to investigate further the underlying biology. Obviously, caution is warranted for the direct translation of animal data to human conditions for a number of reasons. Firstly, as
mentioned above, experiments in this thesis, as well as many other animal studies, used ‘constant’ shift work conditions, without rotational shifts. Adding this factor would improve animal models, at the cost of an increase of labour intensity and complexity to interpret causative relations. Secondly, Chapter 2 described metabolic disruption by shift work conditions and a few categories presented clear differences between mice and rats. Moreover, for instance, effects on body weight, food intake and glucose metabolism were more often observed in mice models, regardless of the manipulating condition, whereas rats responded more frequently by changes in locomotor activity and lipid metabolism. It is unknown what causes these differences, and it raises new questions on how energy homeostasis is species-dependently integrated with the circadian system, since the resemblance of the well-conserved circadian system between rodent species, and between rodents and humans, is large. The same applies for comparisons of glucose metabolism, which is both similar and different between rodents and humans [169]. This is important to realize when translating animal data to human conditions, and stresses the need for human studies. Furthermore, one needs to be aware of the species differences in spectral sensitivity of retinal photoreceptors when studying effects of light on physiology [170, 171]. Unequal importance of rod and cone input to ipRGC signalling for circadian entrainment has been described between mice and rat retinas [170]. Pronounced differences exist between rodents and human photoreception [172, 173], and it has been suggested that rats are a better model to compare with humans than mice [170]. The unequal composition of retinal photoreceptors is perhaps partly explained by the diurnality of humans and nocturnality of most laboratory mice and rats. Though, SCN responses to light have been demonstrated to be largely similar between diurnal and nocturnal species [174]. Also the cFOS rhythm in the SCN is similar in diurnal and nocturnal animals [175]. The nocturnal or diurnal phenotype has been considered to arise from the phenotype of interneurons targeted by the SCN, perhaps through a difference in their excitatory or inhibitory nature [176]. However, this was studied between rodent species and it remains to be studied whether this holds true for human SCN-output pathways, and in what way this may play a role in physiological consequences of, for instance, nocturnal light exposure. At last, importantly, many human shift workers are of the female sex. Sex
differences have been described for circadian characteristics and metabolic susceptibility [177–179], with women suffering more from sleep and circadian disruption by shift work [180]. Still, most animal experiments, including all those in this thesis, are performed with male organisms only. This large flaw might cause a delay in the development of solutions for health problems arising after circadian disturbance, and this aspect deserves attention in future studies. Similarly, the effect of aging is of significance in the tolerance to shift work and circadian stability, leading to an additional factor to be taken into account.

In conclusion, the research in this thesis is ultimately aimed to improve the health of shift workers by describing additional physiological functions that are negatively affected by shift work conditions. We showed that eating at the unusual time-of-day causes negative consequences at different levels of the body, which might be helpful in the design of chrononutrition and advice for shift workers. Furthermore, we showed that light at night rapidly affects glucose metabolism negatively, thus potentially contributing to the high incidence of diabetes type 2. Finally, to a large extent, the presented data can be applied to an even larger part of the world’s population as billions of people are frequently exposed to circadian disruptive conditions, such as environmental light pollution, frequent use of late night artificial lights (by electronic devices), a sedentary and indoor lifestyle, late-night food consumption, crossing of time zones, and social jetlag. The increasing correlations and associations between circadian disruption and health problems further stress the importance to unravel the underlying biology.

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