Shift work, circadian rhythms and the brain

Identifying biological mechanisms underlying the metabolic and cognitive consequences of work timing, using a rat model

Andrea Rørvik Marti

Thesis for the degree of Philosophiae Doctor (PhD)
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Scientific environment

Throughout my time as a PhD-candidate, I have been employed at the Department of Biological and Medical Psychology at the University of Bergen and a member of the Bergen Stress and Sleep Group (BSSG). I have been associated with the International Graduate School in Integrated Neuroscience (IGSIN) and the National Research School in Neuroscience (NRSN).

The experiments in this thesis were carried out at the animal facility and biochemical laboratory at the Department of Biological and Medical Psychology and the laboratory of the Neuroscience Research Group at the Department of Biomedicine at the University of Bergen. The synopsis itself was written up in my home office, in accordance with social distancing guidelines due to the ongoing COVID-19 pandemic.

My main supervisor Professor Janne Grønli is head of the Bergen Stress and Sleep Group at the Department of Biological and Medical Psychology at the University of Bergen. My co-supervisor, Professor Ståle Pallesen, is associated with the Department of Psychosocial Science at the University of Bergen. My co-supervisor Professor Clive R Bramham is head of the Neuroscience Research Group at the Department of Biomedicine at the University of Bergen.

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Abstract

Shift work, and night shift work in particular, is associated with negative health effects. In the short term, night shift work is associated with increased risk of errors and accidents. In the long term, night shift work is associated with metabolic disturbances, including increased risk of obesity and type 2 diabetes. Still, individual tolerance to shift and night shift work varies considerably.

Night shift work challenges the body's normal circadian rhythmicity. Virtually every biological process within the body exhibits daily rhythms. Individual rhythms of cells and tissues are synchronized to the outside world by time cues (zeitgebers). The most prominent zeitgeber is light, but timing of food intake is an important zeitgeber for the metabolic system. The suprachiasmatic nucleus (SCN) of the hypothalamus sets and synchronizes rhythms within individual tissues and cells. Within cells, rhythms are regulated by clock genes and clock proteins. One clock protein, BMAL1, has also been shown to regulate protein synthesis by acting as a promoter of translation initiation.

The mechanisms that underlie the negative health effects of night shift work are not fully understood. Circadian misalignment resulting from altered timing of food intake is thought to underlie much of the long term negative metabolic effects, but the acute effects of shifted timing of food intake are less clear. When it comes to the cognitive disturbance associated with shift work, disturbed sleep (both quantity and quality) has been shown to play a part, but less is known about the role of the circadian clock.

The aims of this thesis are twofold. Firstly, to investigate the acute effects of simulated night shift work on metabolic (paper I) and brain (paper II) functioning. Secondly, to understand how individual factors may predict brain functioning following simulated night shift work (paper III). These aims are addressed using a rat model of shift work. In this model, rats are exposed to forced activity in automatically rotating wheels for 8 hours a day for 3-4 consecutive days, either in the middle of their active phase to simulate human day shift work ("active workers") or in the middle of their rest phase to simulate human night shift work ("rest workers").

In paper I, the effect of, and recovery from, 3-4 consecutive days of simulated night shift work and accompanying shift in the rhythm of food intake on markers of energy balance and liver metabolism are investigated. Food intake, body temperature, and body weight were monitored as markers of energy balance throughout a 4-day shift work protocol and 8-day subsequent recovery and compared to simulated day shift work. After a 5-week washout period, rats were again exposed to simulated shift work for three consecutive days, fasted for two hours, then sacrificed for collection of liver tissue and analysis of liver gene expression, compared to time-matched controls. The results showed dysregulation of markers of energy balance during simulated night shift work, which took more than eight days to recover. Markers of liver energy storage were upregulated, and markers of energy breakdown were downregulated after just three days of simulated night shift work.

In paper II, the effects of simulated shift work on BMAL1-driven translation initiation and related markers within the hippocampus and prefrontal cortex (PFC), brain areas important for cognitive functioning, were investigated. Rats were exposed to three days of simulated shift work, recovered in their home cage for two hours, then sacrificed for collection of brain tissue. Expression of protein markers regulating translation initiation was analyzed using m^7GTP (cap) pulldown and western blot and compared to time-matched controls. Results showed that after simulated night shift work, BMAL1-driven translation initiation was impaired within the PFC, but not the hippocampus, at a time-point when translation initiation is normally promoted.

In paper III, the effects of simulated shift work on cognitive performance on a spatial memory task, the Morris Water Maze (MWM), were first investigated. Rats were taught to identify a hidden platform location before being exposed to three consecutive days of simulated shift work. Immediately after the third shift, recall on the MWM task was tested. Rest workers took longer to locate the hidden platform compared to active workers. However, there were considerable individual differences in MWM performance, and some rest workers performed on par with active workers. Individual differences were also observed in PFC markers of brain protein synthesis. Therefore, hierarchical regression analysis was utilized to test how individual

variation in factors relating to daily rhythmicity, sleep drive, and glucocorticoid levels might predict spatial memory performance and PFC markers of protein synthesis. Results showed that that type of work, as well as individual differences in daily rhythmicity, sleep drive, and serum glucocorticoids, predicted different aspects of spatial memory performance and PFC markers of protein synthesis.

The present findings suggest that just 3-4 days of simulated night shift work is sufficient to disturb metabolic regulation and markers of brain functioning, and that individual variation in a range of predictors relating to circadian rhythmicity and sleep can predict different aspects of brain functioning after simulated shift work. Much is still unknown about the mechanisms that underlie the negative health effects of shift work. The present findings may allow further elucidation of how circadian misalignment impacts all aspects of health, both in those who are engaged in shift work, and in other populations.

List of publications

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Marti, A. R., Pedersen, T. T., Wisor, J. P., Mrdalj, J., Holmelid, O., Patil, S., Meerlo, P., Bramham, C. R. and Grønli, J. (2020). Cognitive function and brain plasticity in a rat model of shift work: role of daily rhythms, sleep and glucocorticoids. *Scientific Reports, 10*(1).

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Abbreviations

4E-BP2	eIF4E-binding protein 2
ACTH	Adrenocorticotropic hormone
Arc	Activity-regulated cytoskeleton-associated protein
ATP	Adenosine triphosphate
AW	Active worker
BMAL1	Brain-and-muscle arnt-like protein 1
ChREBP	Carbohydrate responsive element binding protein
CLOCK	Circadian locomotor output cycles kaput
COMT	Catechol-O-methyltransferase
Cry	Cryptochrome
CYFIP1	Cytoplasmic FMRP-interacting protein 1
DGAT	Diacylglycerol O-acyltransferase
DNA	Deoxyribonucleic acid
EEG	Electroencephalogram
eIF4	Eukaryotic initiation factor 4
EMG	Electromyography
FAS	Fatty acid synthase
FEO	Food-entrainable oscillator
FMRP	Fragile X mental retardation protein
GPAM	Glycerol-3-phosphate acyltransferase 1, mitochondrial
HPA	Hypothalamic-pituitary-adrenal
ipRGC	Intrinsically photosensitive retinal ganglion cells
IRS2	Insulin receptor substrate 2
LD	Light/dark

LTD	Long-term depression
LTP	Long-term potentiation
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
MWM	Morris Water Maze
NREM	Non-REM
p-BMAL1 / eIF4E / S6K1	Phosphorylated BMAL / eIF4E / S6K1
Per	Period
PFC	Prefrontal cortex
PPAR	Peroxisome proliferator-activated receptor
PYGL	Phosphorylase, glycogen, liver
R1-8	Recovery day 1-8
REM	Rapid eye movement
RNA	Ribonucleic acid
RW	Rest worker
S6K1	Ribosomal protein S6 kinase beta-1
SCD1	Stearoyl-CoA-desaturase
SCN	Suprachiasmatic nucleus
SREBP1c	Sterol regulatory element-binding protein 1c
SWA	Slow-wave activity
SWE	Slow-wave energy
SWS	Slow-wave sleep
W1-4	Work-day 1-4
ZT	Zeitgeber time

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1. Introduction

1.1 Shift work

We live in a 24-hour society where services within a range of sectors are required around the clock. Shift work is usually defined by work time arrangements that fall outside the traditional daytime (08.00-18:00) weekday work (Burch et al., 2009; Kecklund & Axelsson, 2016). More than 20% of European workers report working shifts, with the highest proportion of shift workers found within the healthcare (40%), transportation (33%), and industry (28%) sectors (Eurofund, 2017).

Shift work challenges the normal daily rhythm that most species have evolved to follow through the daily cycling of light and dark. The type, duration, timing, and rotation of work hours vary to a large extent. Shift work can be "fixed," i.e., consisting of evening or night work, or rotating, consisting of a schedule varying between morning, evening, and night shifts. The definition of what constitutes a night shift varies but usually involves work that interferes with normal nocturnal sleep (International Agency for Research on Cancer, 2010; Kecklund & Axelsson, 2016).

1.1.1 Cognitive and health consequences of shift work

Shift work is associated with a range of negative consequences, both in the short and in the long term. Night shift work can be particularly detrimental to worker health (Boivin & Boudreau, 2014; Øyane, Pallesen, Moen, Åkerstedt, & Bjorvatn, 2013). In the short term, shift work impairs waking function and cognitive performance and increases the risk of accidents and errors (Folkard & Tucker, 2003). Compared to the morning shift, the risk of errors and accidents is higher on the evening shift and highest on the night shift. Risk also increases across consecutive night shifts worked. Moreover, daytime sleep after a night shift is typically shorter than normal nocturnal sleep (Pilcher, Lambert, & Huffcutt, 2000).

In the long term, shift work is associated with increased risk of multiple health complaints, and illness. Shift work has been reported to increase the risk of gastrointestinal complaints and cardiovascular diseases (Knutsson & Bøggild, 2010;

Torquati, Mielke, Brown, & Kolbe-Alexander, 2018). It is associated with some types of cancer, including prostate and breast cancer (see Kecklund & Axelsson, 2016, for systematic review). Notably, shift work is strongly linked to metabolic disorders, including the risk of developing type 2 diabetes and obesity (Gan et al., 2015; Kecklund & Axelsson, 2016; Strohmaier, Devore, Zhang, & Schernhammer, 2018).

Although shift work at the group level is associated with adverse health outcomes, not all workers experience negative effects to the same extent. The term shift work tolerance has been introduced to describe some workers' ability to adapt to shift work without experiencing adverse effects (Saksvik, Bjorvatn, Hetland, Sandal, & Pallesen, 2011).

1.1.2 Shift work and circadian rhythms

Shift work, especially night shift work, can cause misalignment of the body's internal circadian rhythms. Circadian rhythms are ubiquitous in biological systems and pervade every bodily function. Disturbance to circadian rhythms can disrupt other physiological processes. This is one of the main frameworks to understand how shift work impairs health. However, there are many unanswered questions about the underlying mechanisms. How does the circadian system impact bodily functioning? What happens in the body, and the brain, when an individual is exposed to night shift work? Moreover, why are some individuals more shift work tolerant than others?

The aims of this thesis are twofold: firstly, to investigate the acute effects of simulated night shift work on metabolic (paper I) and brain (paper II) functioning. Secondly, to understand how individual factors may predict brain functioning after simulated night shift work (paper III)

In the introductory section, the concept of circadian rhythms and how circadian rhythms contribute to normal physiological functioning are introduced. Then, some basic physiology of the metabolic system and the brain and synaptic plasticity, and how rhythms impact these systems, are outlined. Next, relevant aspects of the shift work literature, to identify gaps in our understanding of how shift work leads to negative health consequences, focusing on metabolic and cognitive outcomes, within

the framework of circadian misalignment, are summarized. Lastly, the stated aims are returned to, and the hypotheses investigated in this thesis are outlined, based on the reviewed literature.

1.2 Circadian rhythms

In any living system, there are observable repetitive, predictable changes across time. These rhythms can vary from rapid, such as neuronal electrical potentials, to slow, such as the annual flowering of some plants, or reproductive activity of some animal species (Breedlove & Watson, 2013). The most pervasive rhythms are those which exhibit daily repetitions, the so-called circadian rhythms. The word circadian derives from the Latin "circa diem", meaning "approximately one day".

Throughout evolution, the Earth's rotation has imposed predictable daily changes in (most notably) light and dark, to which organisms have adapted. These rhythms allow the prediction of environmental phenomena, yielding a considerable evolutionary advantage, becoming ubiquitous, observable in virtually all biological systems, from fungi to mammals (Buijs & Kalsbeek, 2001).

1.2.1 The nature of circadian rhythms

A key aspect of circadian rhythms is that they persist in constant conditions. In the 1700s, the French scientist Jean-Jacques d'Ortous de Mairan observed that after being placed in a dark cupboard, the mimosa plant continued its daily rhythm of opening and closing its leaves (Foster & Kreitzman, 2017). In the 1950s, Jürgen Aschoff, one of the fathers of modern chronobiology, placed volunteers in underground bunkers for days and weeks without access to information about external time. He observed that while synchrony with the external world was gradually lost, circadian rhythmicity persisted at approximately, but not exactly, 24 hours (Aschoff, 1965). In other words, the rhythm started to *free-run*. The fact that circadian rhythms free-run in constant conditions demonstrates that these rhythms are endogenous, generated by a clock within the body which keeps time, even without exposure to external cues.

Some prominent rhythms

Under normal conditions, the circadian clock is entrained to the daily cycle of light and dark. Some circadian rhythms, like the rest/activity cycle, are readily observable. However, daily fluctuations pervade a range of biological systems, including core body temperature, metabolism, cognition, brain functioning, and many more. Circadian rhythms facilitate daily adaptations to rest and activity. For example, a daily surge in glucocorticoid levels starting before natural wake-up time aids physiological systems to predict and adapt to physical activity (Balsalobre et al., 2000). Conversely, in diurnal species, an evening surge in the circadian rhythm hormone melatonin aids the adaptation to rest and sleep (Claustrat & Leston, 2015).

The rhythm of sleep and wakefulness

Sleep is a naturally recurring physiological state characterized by altered consciousness, reduced sensory and motor activity, and reduced interactions with the surroundings (Lockley, 2012). Thus, it can be relatively easily distinguished from wakefulness. Most species exhibit a prominent circadian rhythm in sleep and wake. The two-process model of sleep regulation posits that sleep is regulated by a circadian and a homeostatic factor (Borbely, 1982). The circadian factor determines the timing and length of the main sleep period, while the homeostatic factor determines the intensity of sleep (Borbely, 1982; Borbely, Daan, Wirz-Justice, & Deboer, 2016).

Separating the involvement of the clock and homeostatic processes on physiological phenomena can be achieved in laboratory conditions through so-called forced desynchrony protocols. Normally, dissipation of homeostatic sleep drive, and descension (and subsequent ascension) of the circadian rhythm, co-occur in the biological night. However, under forced desynchrony conditions, these two processes are forced apart, e.g., by extending the sleep/wake cycle to 28 hours, outside the range of entrainment for the circadian system. The circadian rhythm will then start to free-run, while the homeostatic factor will follow the experimentally imposed pattern of sleep and wake.

Forced desynchrony studies have documented that the different stages of sleep are regulated by different processes. Slow-wave sleep (SWS), the restorative sleep characterized by slow (delta) oscillations in the electroencephalogram (EEG), is primarily homeostatically regulated. The intensity and amount of SWS depend on the duration and intensity of the previous wake period (Dijk, 2009; Dijk & Czeisler, 1995). SWS typically occurs most strongly at the beginning of the sleep period when homeostatic sleep drive is high, and is reduced toward the end of the sleep period when the homeostatic sleep drive is dissipated. On the other hand, rapid-eye-movement (REM) sleep, characterized by rapid, desynchronized waves on the EEG, muscle atonia, and rapid eye movements, is strongly regulated by the circadian factor (Cambras et al., 2007). REM sleep propensity is highest toward the latter half of the sleep period, normally occurring in the early biological morning.

1.2.2 Describing circadian rhythms

Circadian rhythms are incredibly diverse but can be universally depicted as a sinusoidal wave, representing the rise and fall of the biological phenomena under circadian control (Figure 1) (Foster & Kreitzman, 2017).

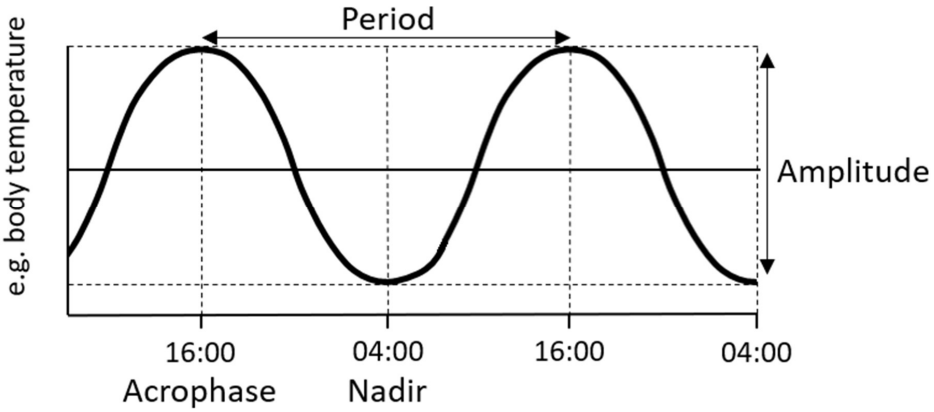

Figure 1. Theoretical schematic representation of the circadian rhythm of, e.g., body temperature. Period, the time to complete one oscillation (here 24 hours). Acrophase, the time of the rhythm peak (here 16:00). Nadir, the time of the rhythm trough (here 04:00). Amplitude, the difference between the peak and trough values. See text for details (section 1.2.2).

The circadian rhythm *period* refers to the time taken for one repetition of the rhythm oscillation (Foster & Kreitzman, 2017). In everyday life, circadian rhythms are entrained to the external 24-hour day and thus exhibit a period of exactly 24 hours. However, the free-running circadian rhythm usually slightly differs from 24 hours, determining individual chronotype (Lack, Bailey, Lovato, & Wright, 2009). Individuals with longer free-running circadian periods tend to have an evening preference, i.e., a late chronotype. Those with shorter free-running periods tend to have a morning preference, i.e., an early chronotype.

The *phase* of the rhythm refers to the placement of the rhythm in time. The time of the rhythm peak is known as the *acrophase*. Conversely, the time of the rhythm trough is termed *nadir*. A change in the timing of the rhythm is referred to as a *phase shift* (Foster & Kreitzman, 2017). As a rule of thumb, one can say that in humans, the body temperature nadir typically occurs about two hours before natural wake-up time (Bjorvatn & Pallesen, 2009).

The *amplitude* of the circadian rhythm refers to the relative height of the curve, i.e., the difference between the trough and the peak value. A high amplitude is thought to indicate a robust circadian rhythm, or strong synchronicity among individual rhythms in cells and tissues (Aschoff & Pohl, 1978; Jafari Roodbandi, Choobineh, & Daneshvar, 2015; Reinberg et al., 1988).

1.2.3 Regulation of circadian rhythms

Circadian rhythms are regulated at different levels. Rhythms exist in individual cells, are encoded in our genes, generated by a core clock within the brain, and (usually) synchronized to the outside world by time-cues known as *zeitgebers*.

The suprachiasmatic nucleus: The core clock

The suprachiasmatic nucleus (SCN), located in the brain's hypothalamus, is the primary driver of circadian rhythms in mammals. It was long suspected to be so since early experiments showed that lesioning the SCN caused a total breakdown of circadian rhythms (e.g., Stephan & Zucker, 1972). Moreover, the rhythmicity of the SCN persisted even after excision from the brain (Groos & Hendriks, 1982). In 1990,

scientists transplanted SCN from hamsters with an abnormally short 20-hour free-running rhythm to hamsters with normal 24-hour rhythms, and vice versa (Ralph, Foster, Davis, & Menaker, 1990). Results showed that recipient animals adopted the free-running rhythm of the donor animals, thus demonstrating that the SCN is the generator of circadian rhythms in the body.

Light: The most potent zeitgeber for the circadian clock

Light is the most potent zeitgeber for the circadian system (Foster & Kreitzman, 2017). In mammals, light stimulates photoreceptors in the eye's retina, obviously of importance for vision (image-forming functions), but retinal photoreception also has several non-image-forming functions (Daneault, Dumont, Masse, Vandewalle, & Carrier, 2016). Axons from cells of the retina signal directly to the SCN via the retinohypothalamic tract (Moore, 1983). Light activates the intrinsically photosensitive retinal ganglion cells (ipRGCs) containing the blue-light sensitive photopigment melanopsin (Freedman et al., 1999; Provencio, Jiang, De Grip, Hayes, & Rollag, 1998; Provencio et al., 2000). Stimulation of ipRGCs by light suppresses the secretion of melatonin, regulates pupil constriction, and activates pathways involved in regulating alertness, cognitive performance, and mood (Daneault et al., 2016; LeGates, Fernandez, & Hattar, 2014). Activation of ipRGCs also allows entrainment of circadian rhythms to the light/dark cycle (Johnson, Elliott, & Foster, 2003; Pittendrigh & Daan, 1976).

The clock in our genes

Circadian rhythms are regulated by a set of genes collectively known as *clock genes*. Konopka and Benzer described in 1971 how mutation to a gene they named Period, or *Per*, induced changes to the circadian rhythm period in the fruit fly, *drosophila melanogaster* (Konopka & Benzer, 1971). More genes that affect the circadian rhythm were eventually discovered, first in drosophila and later also in mammals. In the 1980s, the laboratories of Jeffrey Hall and Michael Rosbash, and Michael Young, showed that the expression of the *Per* gene oscillates across 24 hours (Bargiello, Jackson, & Young, 1984; Reddy et al., 1984). The field eventually came to understand that clock genes are regulated in a transcriptional-translational feedback

loop, which produces circa-24-hour rhythmicity in the genes' expression, see for example, Vosshall and colleagues (1994) and Darlington and colleagues (1998). For these discoveries, Hall, Rosbash, and Young were awarded the 2017 Nobel Prize in Physiology or Medicine.

Clock gene regulation

Clock gene regulation is based on the principles of positive and negative feedback. As the expression of the clock genes brain and muscle ARNT-like 1 (*BMAL1*) and circadian locomotor output cycles kaput (*CLOCK*) increases, BMAL1 and CLOCK proteins are produced. These proteins act as transcriptional activators to promote the expression of the Per and cryptochrome (Cry) clock genes via positive feedback. Per and Cry proteins also act as transcription factors, providing negative feedback on the clock machinery through interaction with *BMALl1* and *CLOCK*, repressing their own expression. Reduced expression of *Per* and *Cry* stops the negative feedback on *BMAL1* and *CLOCK*, allowing expression of *BMALl1* and *CLOCK* to increase once again, starting a new cycle. Due to several fine-tuning and delay mechanisms, one cycle takes approximately 24 hours to complete (King & Takahashi, 2000; Mohawk, Green, & Takahashi, 2012).

This simple and well-characterized clock machinery is evolutionarily ancient, well-preserved across species, incredibly simple, and present in virtually every cell of the body. However, several related functions are still not well understood. For example, it was shown relatively recently that the clock protein BMAL1 not only acts as a transcription factor but can also act as a translation factor to regulate global protein synthesis (Lipton et al., 2015). This finding will be expanded on in later sections.

Clock genes in different tissues

The discovery of clock genes has allowed the study of rhythms in individual cells and tissues. The overall conclusion from this research is that rhythms exist almost anywhere anyone has looked for them (Mohawk et al., 2012). Clock genes impact the physiology and functioning of a given cell, tissue, or organ through tissue-specific clock-controlled genes (Bozek et al., 2009). Bodily functions and systems such as metabolism and cognition are in part regulated by circadian rhythms. The following

sections describe the physiology and function of these systems and how rhythms impact on their functioning.

1.3 Metabolism

The term metabolism refers to any biological process within cells and organisms which work to sustain life. Metabolism is tightly regulated, and can be divided into two main categories; anabolism, the buildup of tissue, and catabolism, the breakdown of tissue (Frayn, 2010). All biological processes require energy, and in all vertebrates, energy is derived through the breakdown of macronutrients (Das & Roberts, 2012). The liver is an essential metabolic organ involved in macronutrient metabolism and adaptations to feeding and fasting.

1.3.1 Energy metabolism

A core aspect of metabolism is the oxidization of nutrients to generate energy required to maintain normal functioning of all bodily processes in the form of adenosine triphosphate (ATP). When ATP is used, heat, carbon dioxide, and water are produced and expended from the body (Frayn, 2010). The unit for energy in foods, the calorie, refers to the amount of heat required to raise the temperature of one gram of water by one degree Celsius. Energy is also important to maintain core body temperature at approximately 37°C (Morrison & Nakamura, 2019).

1.3.2 Energy balance and homeostasis

Many metabolic and other physiologic functions aim to maintain homeostasis, an active process of keeping a physiological process or phenomenon relatively constant (Breedlove & Watson, 2013). In a normally functioning physiological system under normal circumstances, aspects of physiology such as body weight, blood glucose, and body temperature are maintained relatively stable via negative feedback systems. (Breedlove & Watson, 2013).

When energy intake and expenditure are in balance, body weight homeostasis is achieved (Das & Roberts, 2012). A positive energy balance, where energy intake exceeds energy expenditure over time, leads to weight gain. On the contrary, a

negative energy balance with higher energy expenditure than energy intake over time leads to weight loss. A simplified understanding of this suggests that body weight depends only on food ingested and exercise performed. However, this ignores the fact that energy metabolism depends on a wide array of factors, including body composition, the type and quality of the food ingested, the current nutritional status of the body, and, importantly, the timing of food intake (Das & Roberts, 2012). Nevertheless, body weight strongly depends on the amount of energy ingested. Excess energy intake relative to energy use over time may lead to obesity, a condition characterized by excess body fat accumulation that presents a risk to health (Das & Roberts, 2012). Multiple lifestyle-related factors, including short sleep duration, stress, and shift work, have been associated with obesity (Pedersen, Sjödin, & Astrup, 2012).

1.3.3 Role of the liver in metabolism

The liver plays a central role in the metabolism of macronutrients, the parts of food that provide energy to fuel specific metabolic pathways (Frayn, 2010). Carbohydrates and protein products from food are absorbed from the intestine to the portal vein and passed through the liver before entering the general bloodstream (Frayn, 2010). Lipids do not generally enter the bloodstream through this pathway, but the liver is still centrally involved in both storage and release of energy through mechanisms involving lipids. It is also central to the regulation of blood glucose.

Carbohydrates are absorbed in the form of monosaccharides, primarily glucose (Frayn, 2010; Sanders & Lupton, 2012). Glucose is an important energy source for many bodily tissues, and blood glucose is under tight control. The liver is a buffer for glucose. In response to elevated blood glucose, insulin release triggers hepatic storage of glucose in the form of glycogen (Frayn, 2010). When glucose levels are low, stored glycogen is converted back into glucose and released into the bloodstream. Type 2 diabetes is a result of elevated blood glucose caused by insulin resistance (Brand-Miller & Colagiuri, 2012).

The liver has important roles in lipid metabolism, including synthesis, storage, and release of energy from lipids (Frayn, 2010). Most lipids take the form of triglycerides or free fatty acids. Fatty acids can be oxidized in the liver to provide energy for different tissues (Frayn, 2010). Although adipose tissue is the primary storage for triglycerides, the liver can also store fatty acids in this form in response to excess glucose.

Proteins, commonly described as the building blocks of life, are made up of amino acids linked via peptide bonds. In the liver, amino acids enter pathways to produce glucose, fatty acids, and ketone bodies, and importantly, for protein synthesis.

1.3.4 Protein synthesis

A total of 20 amino acids make up all the proteins synthesized by the human body. Proteins can be distinguished based on the number and order of amino acids in the peptide chain (Frayn, 2010). Protein synthesis consists of two main steps; transcription and translation. Transcription occurs in the cell nucleus, where the synthesis of messenger ribonucleic acid (mRNA) occurs by matching complementary bases to the original strand of deoxyribonucleic acid (DNA) (Berg, Tymoczko, & Stryer, 2007). Translation occurs on ribosomes in the cell's cytoplasm, where mRNA is used as a template to build a growing polypeptide chain. Translation initiation is the most highly regulated step of protein synthesis and will be described in detail in section 1.4.4.

Proteins are involved in a wide range of physiological processes, including cell growth, adaptation to changes in the environment, immune functioning, and brain functioning (Pencharz, 2012). Some proteins, such as the clock proteins, can regulate the production of other proteins by acting as transcription or translation factors. Proteins also act as enzymes that trigger enzymatic pathways. They can change their function by the addition of phosphates, so-called *phosphorylation*. This process is in itself regulated by proteins known as protein kinases (Frayn, 2010). One such kinase, the mechanistic target of rapamycin (mTOR), activates pathways involved in regulating translation initiation (Saxton & Sabatini, 2017). mTOR can in turn be

activated by some amino acids, as well as growth factors or other substances signaling stress or hypoxia. In these complex ways, proteins are key in the maintenance of normal functioning throughout the body.

1.3.5 Role of glucocorticoids in metabolism

Glucocorticoids, most notably cortisol in humans and corticosterone in rodents, are steroid hormones released via the hypothalamic-pituitary-adrenal (HPA) axis (Breedlove & Watson, 2013). The release of glucocorticoids is commonly associated with stress, any stimulus or experience that threatens homeostasis (Kinlein & Karatsoreos, 2020), but has a wide range of physiological effects. Metabolically, the effects of glucocorticoid release relate to the rapid mobilization of nutrients to facilitate energy release, such as stimulating gluconeogenesis, allowing for the synthesis of glucose from non-carbohydrate sources (Frayn, 2010). Glucocorticoid release also triggers the mobilization of lipids from adipose tissue and the breakdown of proteins (Akalestou, Genser, & Rutter, 2020). This may make sense in situations of acute stress, but in cases of chronic stress, it may contribute to enhance the risk of metabolic dysregulation and disease (Vegiopoulos & Herzig, 2007).

1.3.6 Rhythms in metabolism

The metabolic system is highly rhythmic. The SCN has direct and indirect anatomical connections to several areas involved in regulating appetite, energy expenditure, and behavioral activity within the hypothalamus and other regions of the central nervous system and peripheral organs (Kalsbeek et al., 2006). Several metabolic compounds involved in the regulation of nutrient and energy metabolism are rhythmic and under control of the circadian clock. Some of these compounds also feed back on the circadian system itself (Panda, 2016).

While many of the body's cells and tissues require a steady stream of energy, energy intake usually occurs in meals, with daily periods of fasting in-between. Thus, a central role of the metabolic system is to facilitate the daily adaptations to feeding and fasting. Rhythms in the liver drive these daily adaptations. Additionally, rhythms

in hormones like glucocorticoids aid in the temporal organization of events involved in the breakdown, uptake, storage, and release of nutrients and related compounds.

Adaptations to feeding and fasting

About 15% of liver transcripts are rhythmic, making it a highly rhythmic organ (Vollmers et al., 2009; Zhang, Lahens, Ballance, Hughes, & Hogenesch, 2014). The liver circadian rhythm is fundamental in glucose homeostasis, counteracting the daily rhythm of feeding and fasting. The liver clock regulates the conversion of glycogen to glucose in the fasted state (Lamia, Storch, & Weitz, 2008). Oscillation of clock genes is also in part involved in regulation of lipid metabolism, including the oxidization of fatty acids within the liver to release energy (Gachon et al., 2011; Le Martelot et al., 2009). Additionally, insulin sensitivity has been shown to vary throughout the biological day (Kalsbeek & Strubbe, 1998). Circadian factors also regulate the release of pancreatic insulin in response to a meal, triggering several processes that promote glucose utilization and storage and inhibit lipid oxidation (Marcheva et al., 2010; Sadacca, Lamia, deLemos, Blum, & Weitz, 2011).

Rhythms in glucocorticoids

About 15% of human blood plasma and saliva contains rhythmic metabolites (Dallmann, Viola, Tarokh, Cajochen, & Brown, 2012). These include components such as fatty acids and amino acids. Glucocorticoids are also rhythmic, rising in the early biological morning, facilitating adaptation to behavioral activity (Kalafatakis, Russell, & Lightman, 2019). Moreover, glucocorticoid response elements on the *Per* gene are involved in regulating appetite by influencing levels of the satiety hormone leptin, and by regulation of blood glucose by influencing the insulin response (So, Bernal, Pillsbury, Yamamoto, & Feldman, 2009). Thus, glucocorticoids regulate metabolic processes via the clock both directly and indirectly

Food is an important zeitgeber for the metabolic system

Feeding activity is under clock control through several pathways, including direct signaling from the SCN to the arcuate nucleus of the hypothalamus, regulating appetite (Kalsbeek et al., 2006). While the SCN is strongly coupled to the light/dark

cycle, the metabolic system is primarily entrained by food (Schibler, Ripperger, & Brown, 2003). Thus, food represents both input and output to the clock. When food availability is constricted to a short time window in the middle of the rest phase, metabolic organs will adapt to the feeding rhythm, while the SCN maintains synchronicity with the light/dark cycle (Atger et al., 2015; Damiola et al., 2000; Satoh, Kawai, Kudo, Kawashima, & Mitsumoto, 2006; Stokkan, Yamazaki, Tei, Sakaki, & Menaker, 2001; Vollmers et al., 2009). Thus, the organism enters a state of *internal desynchrony* or *circadian misalignment*.

The finding that food is a strong zeitgeber for the metabolic system has led to the concept of the *food-entrainable oscillator* (FEO) (Stephan, 2002). Early work assumed that the FEO would be located in a specific brain region, similar to the more studied light-entrainable oscillator in the SCN (Mistlberger, 1994). However, researchers have not been successful in identifying a single location for the FEO. Instead, it appears that the FEO is controlled by a more complex system of oscillators situated both in the brain and in the periphery (Mistlberger, 2011; Pendergast & Yamazaki, 2018).

Rhythm disturbance is detrimental to metabolic health

The maintenance of metabolic rhythmicity is of great importance to metabolic health. In a seminal 2005 paper, Turek and colleagues showed that *CLOCK* knock-out mice (with a genetic mutation turning off the *CLOCK* gene) displayed blunted feeding rhythms, overeating, obesity, and elevated blood glucose. Subsequent studies have shown that disrupting the clock in specific metabolic tissues, including the liver or pancreas, is sufficient to induce metabolic disturbances such as dysregulation of blood glucose homeostasis (Marcheva et al., 2010; Pan, Bradfield, & Hussain, 2016). The link between the clock and metabolic dynamics have since been studied in both human and animal laboratories and profoundly impacted shift work research, to be further described in section 1.5.2.

1.4 Cognition, the brain, and synaptic plasticity

1.4.1 Cognition and the brain

Cognition is a term in psychology broadly used to describe processes involving the acquisition of knowledge and understanding through thought, experience, and the senses (Breedlove & Watson, 2013). Cognitive performance or ability often refers to the successful achievement of these processes. Cognition works to allow the individual or animal to solve problems and adapt to changing environments.

Tests of cognitive performance have been developed to gauge specific aspects of cognition, such as working memory, long-term memory, spatial memory, attention, response inhibition, and pattern recognition. Spatial tasks have been mainly used in the animal literature to assess several cognitive abilities, including spatial learning (acquisition) and memory (Brandeis, Brandys, & Yehuda, 1989). These tasks, such as the Morris Water Maze (MWM) task, have also been directly linked to the functioning of specific areas of the brain, primarily the hippocampus.

The hippocampus is the most studied brain area for cognition and essential in encoding long-term memory and spatial navigation and awareness (Clark et al., 2019; Whitlock, Heynen, Shuler, & Bear, 2006). Another brain area involved in cognitive performance is the prefrontal cortex (PFC). The PFC is important for higher-order, or executive, cognitive functions, including processes like directing and maintaining attention, planning, working memory, inhibition, error monitoring, and problem-solving (Funahashi & Andreau, 2013; Miller, 2000).

1.4.2 The neuron and the synapse

The human brain contains about 100 billion (10^{11}) neurons, structurally, metabolically, and functionally separate cells that communicate across synapses (Breedlove & Watson, 2013). The number of synapses in the brain is not constant. In the human brain the number of synapses peaks early in development, with maximal synapse density at around two years of age (Huttenlocher, 1979). Subsequently, the brain undergoes so-called competitive activity-dependent synaptic pruning, where synapses that are not in use are eliminated, reducing synapse density by about 50%

by adulthood (Piochon, Kano, & Hansel, 2016). Synapses are also capable of change in the short term, a phenomenon known as *synaptic plasticity*.

1.4.3 Synaptic plasticity

The term synaptic plasticity broadly refers to the ability of a synapse to change in strength in response to use or disuse (Nikolaienko, Patil, Eriksen, & Bramham, 2018). These processes can modify the activity and organization of specific brain areas and circuits. Ramon y Cajal suggested in 1894 that memories could be stored by changes in the strength of connections between neurons. This notion was further advanced in the 1940s by Donald Hebb, who suggested that simultaneous activation of pre and postsynaptic membranes, resulting in lasting increased connection strength between neurons, is what underlies learning and memory. The theory of Hebbian plasticity (what will herein be referred to as synaptic plasticity) is commonly summarized by the saying “cells that fire together, wire together”.

Hebbian plasticity can be modeled by providing electrical stimulation to specific groups of cells, and recording the responding change in synaptic potential in a group of receiving cells. A lasting increase in synaptic potential is referred to as long-term potentiation (LTP) (Lynch, 2004). LTP has been mainly studied within the rodent hippocampus as a model for studying the biochemical substrates that underlie synaptic plasticity. Tasks that engage the hippocampus, such as a spatial task, are thought to elicit LTP-like responses within specific compartments of that brain area (Whitlock et al., 2006). Strengthened synapses can be returned to a baseline activity level through a negative feedback process known as synaptic scaling (Turrigiano, 2008). A related phenomenon, long-term depression (LTD), refers to a lasting reduction in synaptic potential (Piochon et al., 2016). Together, these are the most studied forms of long-term synaptic plasticity.

An important process in synaptic plasticity is the transcription of immediate-early genes such as the activity-regulated cytoskeleton-associated protein (Arc) (Nikolaienko et al., 2018). In the neuron, Arc mRNA is transported to, and translated in, regions where synapses have been activated recently (Bramham et al., 2010).

Sustained Arc translation at the synapse across a time-frame of several hours is required for the formation of late-phase LTP (Messaoudi et al., 2007). Disrupted Arc functioning has been implicated in several disease states linked to cognition, such as Alzheimer's disease (Kerrigan & Randall, 2013) and memory loss (Gautam, Wadhwa, & Thakur, 2013).

De novo protein synthesis and protein translation

In long-term synaptic plasticity, persistent changes in synaptic strength lasting more than 3-4 hours require de novo protein synthesis. Before it is exported from the nucleus, the mRNA transcribed from DNA undergoes a process known as "capping," where a guanine nucleotide is attached to the 5' end of the mRNA strand via a triphosphate group. This process has many functions, but of importance to this thesis, it allows recognition of the mRNA to initiate translation (Ramanathan, Robb, & Chan, 2016).

Translation consists of three steps; initiation, elongation, and termination (Hershey, Sonenberg, & Mathews, 2012). Translation initiation is the most highly regulated step of protein synthesis and strongly implicated in synaptic plasticity (Bramham, Jensen, & Proud, 2016; Siddiqui & Sonenberg, 2015). Translation initiation requires several so-called eukaryotic initiation factors (eIFs) to facilitate the formation of a translationally competent ribosome (Bramham et al., 2010). The phosphorylated initiation factor eIF4E (p-eIF4E) binds to the mRNA cap and recruits binding partners like eIF4G and eIF4A to form the initiation complex eIF4F, allowing translation to occur (Gal-Ben-Ari et al., 2012; Gkogkas, Sonenberg, & Costa-Mattioli, 2010) (Figure 2A).

Interaction of cap-bound eIF4E with eIF4G can be inhibited by repressors, the eIF4E-binding proteins (4E-BPs), such as 4E-BP2, or the cytoplasmic fragile X mental retardation-interacting protein 1 (CYFIP1) and its binding partner the fragile X mental retardation protein (FMRP). These proteins prevent eIF4F complex formation, and hence represses translation initiation (Bidinosti et al., 2010; De Rubeis et al., 2013; Napoli et al., 2008) (Figure 2B). The repression can be alleviated by ERK

signaling to MNK which phosphorylates 4E and triggers removal of repressors, or by mTOR activation which phosphorylates 4E-BP and triggers its release from eIF4E (Laplante & Sabatini, 2009). Dysfunction in the translation initiation machinery is associated with disruption to cognitive function and intellectual disability (Bramham et al., 2016; Curatolo, Moavero, & de Vries, 2015). The circadian clock also directly impacts this machinery, as will be returned to toward the end of the following section.

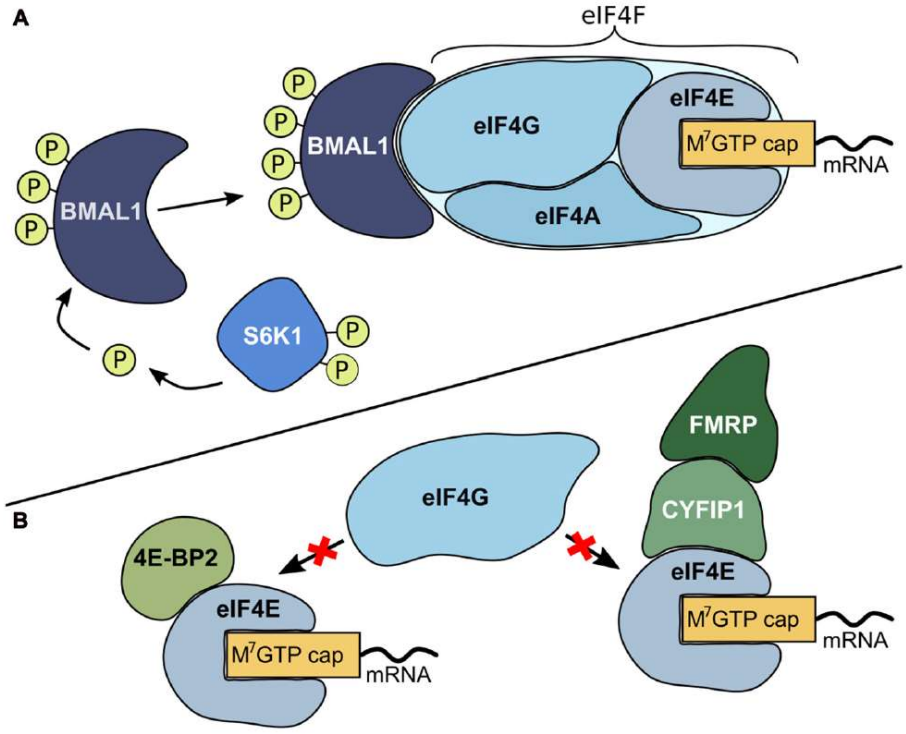

***Figure 2**. Mechanisms for the regulation of translation initiation. (A) Promotion of translation. Translation initiation requires the formation of the eukaryotic initiation complex eIF4F. The circadian clock protein BMAL1 can be phosphorylated by S6K1 downstream of mTOR. Phosphorylated BMAL1 binds to the eIF4F to further enhance translation initiation. (B) Repression of translation. eIF4E-binding proteins can bind to the initiation factor eIF4E, blocking the formation of the eIF4F complex, repressing translation initiation. See text (section 1.4.3-1.4.4) for details. Figure reprinted from paper II (Marti et al., 2017), with permission.*

1.4.4 Rhythms in cognition and plasticity

Circadian rhythms profoundly impact cognition, the brain, and synaptic plasticity. In humans, rhythms in cognition are strongly tied to the daily rhythm of rest and activity (Foster & Kreitzman, 2017). Cognitive performance tends to be better during the active phase than the rest phase (Carrier & Monk, 2000; Foster & Kreitzman, 2017). However, cognitive performance is influenced by both circadian and homeostatic processes (Van Dongen & Dinges, 2005). Forced desynchrony experiments suggest that performance on a range of cognitive tasks follows the rhythm of body temperature (Folkard, Wever, & Wildgruber, 1983; Monk et al., 1983). However, under entrained conditions, there are several peaks in cognitive performance, the timing of which depends on the nature of the task (Carrier & Monk, 2000).

Circadian rhythmicity can also be detected within the brain. Clock genes oscillate in virtually every brain area examined, including areas important for cognitive performance such as the hippocampus and the prefrontal cortex (Li et al., 2013; Seney et al., 2019). Clock gene expression oscillates in all six layers of the mouse cerebral cortex (Rath, Rohde, Fahrenkrug, & Moller, 2013). Interestingly, while these clocks are under central clock control, the phase of clock gene oscillation in the mouse cortex is delayed by approximately 5 hours compared to the SCN (Rath, Rovsing, & Moller, 2014). One study in humans using functional magnetic resonance imaging (fMRI) showed that in response to repeated cognitive tasks, multiple brain areas, including the basal ganglia and thalamus, respond differently depending on the time-of-day, in a circadian pattern (Muto et al., 2016).

Rhythms in synaptic plasticity

The circadian clock is also involved in the regulation of synaptic plasticity. Circadian rhythms in a synapse's propensity or ability to undergo synaptic plasticity can be considered a form of *metaplasticity* (Abraham & Bear, 1996). For example, rhythms in LTP induction has been demonstrated in many species (Barnes, McNaughton, Goddard, Douglas, & Adamec, 1977; Chaudhury, Wang, & Colwell, 2005). Additionally, the density of dendritic spines changes across the day in a circadian fashion (Jasinska et al., 2015; Jasinska et al., 2019). A recent study showed that,

while only 6% of forebrain mRNA is rhythmic, 67% of forebrain *synaptic* mRNAs were rhythmic (Noya et al., 2019). Additionally, the phosphorylation state of 30% of proteins within forebrain synapses was found to be rhythmic (Bruning et al., 2019). The rate of protein synthesis in the brain also exhibits circadian variations, generally peaking around the middle of the active phase (Ayers, Kapas, & Krueger, 1996).

mTOR and the translation initiation machinery are implicated in circadian adaptations and regulated by the circadian clock. mTOR activation and subsequent eIF4E-phosphorylation are involved in SCN adaptations to the light/dark cycle (Cao et al., 2015; Cao et al., 2013). Phosphorylation of S6K1 and 4E-BP1, downstream of mTOR, is rhythmic within the SCN (Cao et al., 2013). mTOR and downstream factors such as eIF4E and 4E-BPs have been shown to oscillate within hippocampal, hypothalamic, and frontal regions of the brain (Albert, Cornu, & Hall, 2015; Khapre et al., 2014; Saraf, Luo, Morris, & Storm, 2014). Importantly, as mentioned in the previous section, the circadian clock has itself been directly implicated in the regulation of translation initiation.

The role of *BMAL1* in clock gene regulation is well characterized. *BMAL1* is itself a clock gene, involved in regulating the transcription of other clock genes within the cell nucleus. It has also been known for some time that the clock protein BMAL1 is shuttled into and out of the cell's cytoplasm in a circadian fashion (Kwon et al., 2006; Tamaru et al., 2003). However, the role of BMAL1 in the cytoplasm was not known until a 2015 study showed that BMAL1, phosphorylated by S6K1, binds to the eIF4F complex and enhances global protein translation (Lipton et al., 2015) (Figure 2A). Thus, BMAL1 not only acts as a transcription factor in the cell's nucleus but also as a translation factor in the cell's cytoplasm, involved in regulating the rate of protein synthesis.

1.5 A brief history of shift work research

The shift work literature is vast. A search for "shift work" OR "night work" performed in September 2020 in Pubmed, the archive of the U.S. National Institutes

of Health's National Library of Medicine, yielded nearly 40,000 results. Although the search is imprecise, the results can provide an overview of the historical development in the research activity in the field (Figure 3). Before 1980 there was a total of 643 publications. The number of published papers has since risen exponentially, to a total of 1,645 in the 1980s, about 4,000 in the 1990s, 9,000 in the 2000s, and 25,000 in the 2010s alone.

It is impossible to fully summarize and review the complete shift work literature. However, this section will provide some historical perspective to give an idea of the trajectories that this area of research has traversed. By placing the field in a historical perspective, it might be feasible to make some predictions about the future of the field and how this thesis fits within it.

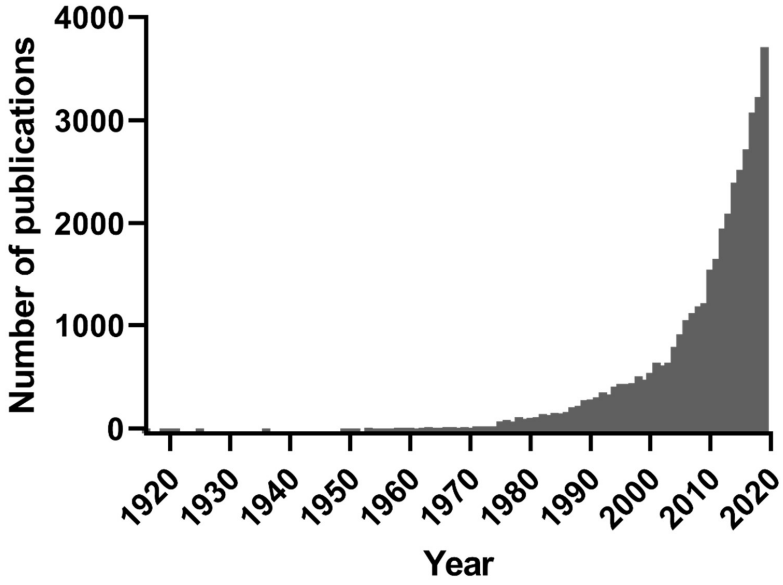

Figure 3. The number of publications yielded from the search terms "shift work" OR "night work" on the database for the archive of the U.S. National Institutes of Health's National Library of Medicine (PubMed), per year. The search was performed in September 2020.

1.5.1 Early shift work history

Shift work has existed at least since the dawn of industrialization. Early legislation regarding who could and who could not perform shift work (specifically night shift work) suggests that the harmful effects of these types of work were recognized already in the early 1800s. The British Health and Morals of Apprentices Act of 1802 made night shift work illegal for apprentices (referenced in U.S. Department of Labor, 1919). In 1833, the Factory act made night shift work illegal for children under the age of 18 (referenced in U.S. Department of Labor, 1919). It appears these laws were not strongly enforced, but the fact that they were passed speaks to some societal concern for the health effects of night shift work.

Night shift work started to be recognized as a medical and societal problem within the medical literature once women started entering the workforce. The first article appearing on PubMed was published in 1916, entitled "Hours of work for women: night work for nurses and munition workers." The article appears as an editorial, with no author credited. As the World War I had sent men to the battlefield and women to work in the factories, there were concerns about the effects of night shift work on women's health. Of adverse health effects, "fatigue," "liability to accident," and "wastage" were mentioned. It was recommended that "two months at a time is quite long enough for any hospital nurse to remain on night duty, and that in no circumstances should three months at a stretch ever be exceeded". Today's recommendations are slightly more conservative: In Europe it is typically recommended to restrict the number of consecutive night shifts to two or three (Burgess, 2007). However, the health problems listed are still of concern today.

A 1919 paper in the American Journal of Public Health entitled "medical argument against night work especially for women employees" echoes the sentiments, listing several factors contributing to the detrimental health effects of night shift work, including disturbed sleep, irregular eating patterns, risk of accidents and errors, fatigue and illness. The author concludes that "night work for women is not in line with conserving womanhood or childhood. It is a menace to state and national vitality" (Hayhurst, 1919).

One of the earliest scientific studies of shift work comprised an observational study examining subjective fatigue in factory workers across a standard 8-hour daytime shift (Griffith, Kerr, Mayo, & Topal, 1950). The authors found that fatigue was lowest at the beginning of the shift and after the lunch break, and highest just before lunch and at the end of the shift. The early literature is dominated by opinion articles and anecdotal reports and small scale observational studies, examining topics like the effects of shift type on psychological and physiological markers of stress (Groll & Haider, 1965) and subjective well-being (Dirken, 1966).

From the 1950s onward, there was an increased interest in how shift work contributed to circadian rhythm disturbance and how this, in turn, impacts health. This turn coincides with the rise of the field of chronobiology. American scientist Curt Richter had shown already in the 1920s that the rat rest/activity rhythm persisted in constant conditions (Richter, 1922). In the 1950s and onward, researchers like Jürgen Aschoff and Colin Pittendrigh systematically studied circadian rhythms in both humans and animals, paving the way for the study of circadian rhythms and their relation to shift work and various aspects of health (Aschoff, 1960; Pittendrigh, 1960).

1.5.2 Shift work and metabolism

Scholars exhibited concern with the food habits of shift workers from early on. One early publication based on a small sample of five shift workers showed that while the total caloric intake was not altered by shift work, there was a change in eating patterns, increased snacking, and preference for foods rich in carbohydrates (Reinberg et al., 1979). Interestingly, despite the small sample size, this paper's main conclusions remain supported by the literature today. Shift workers do alter their eating habits; they snack more and make poorer food choices, rich in refined carbohydrates and saturated fats (Lowden, Moreno, Holmback, Lennernas, & Tucker, 2010), but without an increase in total caloric intake (see Bonham, Bonnell, & Huggins, 2016 for meta-analysis).

The systematic study of the associations between shift work and adverse metabolic outcomes did not fully start until the 1980s, and most of the work on this topic was

conducted after the year 2000. Collectively, the literature provided strong evidence of the link between shift work and adverse metabolic outcomes. Meta-analyses have confirmed that shift work, particularly including night work, is associated with an increased risk of metabolic syndrome (Rivera, Akanbi, O'Dwyer, & McHugh, 2020; Wang, Zhang, et al., 2014), type 2 diabetes (Gan et al., 2015; Gao et al., 2020), and cardiovascular events (Torquati et al., 2018; Vyas et al., 2012).

In parallel, the study of chronobiology at the level of basic science had led to the understanding of the intricacies of the molecular clock described in section 1.2.3. Knock-out studies had shown that deletion of specific clock genes led to metabolic disruption (see section 1.3.6). This also made its way into the shift work literature. A 2014 study in humans showed that energy expenditure during daytime sleep after a simulated night shift in laboratory conditions was reduced by about 15% compared to nocturnal sleep following the day shift, providing a potential link between shift work and obesity risk, despite a lack of increase in caloric intake (McHill et al., 2014). A 2008 study had shown similar results using a rat model where rats were exposed to simulated night shift work by forced activity in automatically rotating wheels during the rest phase, five days a week for five weeks. This caused a shift in the timing of food intake, excessive weight gain, and dysregulated rhythm of circulating glucose, corticosterone, and triglycerides (Salgado-Delgado, Angeles-Castellanos, Buijs, & Escobar, 2008). However, when food was made available only in the natural active phase, none of these adverse metabolic effects were observed (Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010). Experimental studies in humans also show that eating on the night shift impairs glucose response to breakfast (Banks et al., 2015) and that this effect is modulated by meal timing (Grant et al., 2017).

Even more recently, the rise of the field of metabolomics has allowed the study of a large number of metabolites and how these change in response to simulated night shift work in humans. One such study showed that when exposed to simulated night shift work, metabolites that were initially found to be rhythmic in blood exhibited reduced rhythm amplitude (Kervezee, Cuesta, Cermakian, & Boivin, 2018). This was

particularly the case for metabolites involved in immune functioning, but also for metabolites involved in protein synthesis. Another study showed changes to rhythmic metabolites associated with adverse blood glucose regulation (Skene et al., 2018). Interestingly, attention is also being brought to how these metabolomic dynamics are altered at the individual level (Kervezee, Cermakian, & Boivin, 2019), increasing the understanding of how individual factors contribute to shift work tolerance.

1.5.3 Shift work, cognition, and brain functioning

Shift work, and night shift work in particular, is associated with increased risk of accidents and errors. However, the risk is not constant across the course of the night shift. In 1923, Vernon noted that risk increases across the first few hours of the night shift (referenced in Folkard & Tucker, 2003). In a meta-analysis, Folkard and Tucker (2003) summarize ten studies that suggest the same pattern; risk increases in the first few hours, before falling and reaching a minimum in the morning at the end of the shift. Similar patterns have been observed in experimental studies. During a night of sleep restriction, cognitive performance gradually worsens across the course of the night but then improves in the early morning (Van Dongen & Dinges, 2005).

Shift work challenges not only the circadian, but also the homeostatic system. Night shift work is associated with decreased sleep length relative to day shift, and shift workers report poorer sleep quality compared to day workers (Pilcher et al., 2000; Short, Agostini, Lushington, & Dorrian, 2015; Torquati et al., 2018). Sleep loss is associated with cognitive deficits on a range of tasks, particularly impairments in task performance speed and accuracy, impaired vigilance and working memory, and long-term memory (Alhola & Polo-Kantola, 2007; Lim & Dinges, 2010). Night shift work is also associated with incidence of micro-sleep episodes and intrusion of EEG slow-waves during wakefulness, associated with drowsiness in both human and animal studies (Grønli et al., 2017; Liang et al., 2019). These effects can be particularly detrimental in situations such as the commute home after the night shift due to a strong circadian drive for sleep (depending on the time of day) and simultaneous high homeostatic sleep pressure after prolonged wakefulness, potentially combined with physical or mental exhaustion after a work shift (Horne & Reyner, 1999; Liang et al.,

2019). Altering the timing or quality of light exposure may mitigate these adverse effects (Sunde, Mrdalj, et al., 2020; Sunde, Pedersen, et al., 2020). Thus far, the vast majority of studies examining the effects of shift work on cognition or brain functioning have been performed in humans. However, one study in rats showed that surprisingly, five weeks of simulated night shift work did not impair performance on an instrumental task compared to simulated day shift work (Leenaars et al., 2012).

Shift work, circadian disturbance, and synaptic plasticity

Very few studies investigate the effects of shift work on brain structure or function at the biochemical level. However, many studies have examined the effects of related phenomena such as circadian misalignment, sleep deprivation, and stress on the brain. Circadian misalignment is detrimental to brain functioning and impairs synaptic plasticity. Knock out of various clock genes results in defects in learning and spatial memory and hippocampal LTP formation (Kondratova, Dubrovsky, Antoch, & Kondratov, 2010; Rawashdeh et al., 2014; Wang et al., 2009). One study showed that chronic circadian disturbance reduced performance on a cognitive task and decreased dendritic length in the medial prefrontal cortex neurons in mice (Karatsoreos, Bhagat, Bloss, Morrison, & McEwen, 2011). These studies provide a basic understanding of how the brain may be influenced by circadian rhythm disturbance. Sleep deprivation and chronic stress also have detrimental effects on brain functioning. The rhythm of circulating glucocorticoids is disturbed after night shift work (Caufriez et al., 2002; Goichot et al., 1998; Harris et al., 2010). The effects of sleep and stress on the brain also interact in complex ways (reviewed in Grønli, Soulé, & Bramham, 2014). Shift work is particularly complex in that it involves disruption to many different systems at the same time. Thus far, few studies examine the effects of shift work on basic brain functioning. Perhaps it is time.

1.5.4 Shift work tolerance and individual differences

The term shift work tolerance entered the literature by the end of the 1970s. Early publications were concerned with the link between body temperature rhythm amplitude and shift work tolerance, suggesting that a strong rhythm amplitude was related to higher tolerance (Andlauer, Reinberg, Fourre, Battle, & Duverneuil, 1979;

Reinberg et al., 1980). Later, much of the literature has been concerned with individual factors such as personality traits and their relation to shift work tolerance (Saksvik et al., 2011). It has long been understood that there are many factors, both internal and external, that impact how well shift work is tolerated by an individual (Axelsson, Åkerstedt, Kecklund, & Lowden, 2004). Recently, there have been attempts to systematize this (Kecklund & Axelsson, 2016), but the majority of shift work research is still concerned with averages across wider groups of workers. For some, the effects are probably far worse than what the literature suggests. These individuals are likely to quit shift work relatively soon, and unlikely to volunteer for experimental studies on shift work, and thus do not contribute much to the overall available data on the health effects of shift work. Those who are left to participate are those who tolerate shift work relatively well. This concept is known as the "healthy shift worker effect" (Knutsson, 2004).

1.5.5 Animal models of shift work

Animal models of shift work have central importance to this thesis, and some general points must therefore be made. The vast majority of research in the field has been published post-2000. Human shift work involves changes to the timing of sleep and wakefulness, physical and social activity, and meals. While the Earth's rotation maintains regular rhythmicity, the timing of exposure to artificial light is changed by shift work. Many animal studies therefore attempt to model shift work by changes in the timing of light exposure, but these models more resemble another form of circadian rhythm disturbance, namely jet lag. In recent years, the most prominent type of animal model involves forced activity by automatically rotating wheels during the rest phase. At least three different laboratories employ such methods to model shift work in rats (Grønli et al., 2017; Leenaars et al., 2012; Salgado-Delgado et al., 2008). In this model, food is freely available (*ad libitum*), and animals are kept awake and active in slowly rotating wheels. Most laboratories use a protocol where animals "work" for 8 hours in the middle of their rest phase or their active phase to simulate night shift work or day shift work, respectively. In-between shifts, the animals are allowed to rest. Light is maintained at a standard light/dark (LD) cycle with 12 hours of lights on and 12 hours of lights off. Thus, the SCN receives a daytime signal that

conflicts with the imposed rhythm of activity and rest. The rhythm of food intake tends to gradually shift across a few days before stabilizing to follow the rest/activity rhythm.

Most animal studies on the effects of shift work focus on metabolic effects (see Opperhuizen, van Kerkhof, Proper, Rodenburg, & Kalsbeek, 2015, for review). There has been much less focus on brain functioning, including cognition. One rat study showed desynchrony between hypothalamic brain regions, the SCN remaining synchronized to the LD cycle, and regions involved in metabolic regulation becoming synchronized to the shifted feeding/fasting rhythm, after simulated night shift work (Salgado-Delgado 2010). Another showed no significant effects of simulated night shift work on an instrumental learning task (Leenaars et al., 2012). These studies have all focused on long-term effects across a timeframe of five "workdays" per week, for five weeks. A study from the present laboratory showed degraded waking function as evidenced by enhanced slow-wave activity during wakefulness, as well as shifted sleep timing, within just four days of simulated night shift work, compared to simulated day shift work (Grønli et al., 2017). Apart from this study, acute effects appear to have not been considered.

1.5.6 Identifying the biological mechanisms underlying the adverse health effects of shift work

The last decades have seen an explosion in basic science, allowing the understanding of e.g., the functionality of the circadian system down to the level of the gene. However, there is much to be learned about how rhythm disturbance and shift work impact health. Shift work impacts multiple physiological systems and impairs rhythms, metabolism, and waking function. Essentially, shift work has detrimental effects on virtually every biological system, and it is influenced and modulated by a significant number of factors. It will be a long time before the understanding of how shift work affects health is complete. Relatively much is known about the overall long-term effects of shift work on health (particularly metabolic health), but far less is known about the acute effects.

It is an aim in science to make generalizable conclusions. In general, shift work is associated with an increased risk of adverse health effects. However, this is only part of the story, as the observed effect is an average on the group level. For some, the effects are probably far worse than the literature suggests due to the healthy shift worker effect. However, rather than averaging out individual variations, it is possible to take advantage of these variations to explain which individual characteristics predict tolerance to shift work. Statistical tools allow the generation of further testable hypotheses. It is an attractive idea that future research may generate knowledge to predict who will tolerate shift work well and who will not. This thesis might make a (however small) contribution toward this step.

1.6 Aims and hypotheses

In the present work, a rat model of shift work with forced activity is used to achieve the following overall aims:

Aim 1: To investigate the acute effects of simulated shift work on metabolic (paper I) and brain (paper II) functioning.

Aim 2: Understand how individual factors may predict brain functioning following simulated shift work (paper III)

Paper I

Several human and animal studies have investigated the effects of shift work on the metabolic system. However, the acute metabolic effects of night shift work and shifted rhythm of food intake on metabolic functioning are not well understood. In paper I, the effects of, and recovery from, 3-4 consecutive days of simulated night shift work and accompanying shift in the rhythm of food intake on markers of energy balance and liver metabolism, compared to simulated day shift work, are investigated. It is hypothesized that 3-4 days of simulated night shift work is sufficient to temporarily disturb metabolic regulation and that this disturbance will take up to eight days to recover.

Paper II

Night shift work is associated with an increased risk of errors and accidents and reduced cognitive performance. However, little is known about how night shift work affects brain functioning and synaptic plasticity-related processes. The circadian clock protein BMAL1 directly impacts the regulation of protein translation. In paper II, the effects of three consecutive days of simulated shift work on BMAL1-driven translation initiation and related markers in the hippocampus and prefrontal cortex, brain areas known to be important for cognitive functioning, are investigated. It is hypothesized that three consecutive simulated night shifts are sufficient to disrupt the translation initiation-machinery in these brain areas.

Paper III

It is not clear whether simulated night shift work impairs cognitive performance in rats. Therefore, in paper III, spatial memory performance is tested after three consecutive days of simulated shift work. It is hypothesized that this is sufficient to impair spatial memory performance in simulated night shift workers. Also, it is known that there are considerable individual differences in shift work tolerance. Therefore, whether such individual differences in daily rhythmicity, sleep drive, and serum glucocorticoids (corticosterone) in response to simulated shift work, predicts spatial memory performance and protein markers of prefrontal cortex functioning after simulated shift work is investigated. It is hypothesized that both spatial memory performance and protein markers of brain functioning can be predicted by individual variation in rhythmicity, sleep, and corticosterone.

2. Methods

For detailed descriptions of procedures, protocols, equipment, drugs, and reagents, see the methods sections of papers I, II, and III.

2.1 Ethics, animals, and housing

This study was carried out in accordance with Norwegian laws and regulations, and The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The protocol was approved by the Norwegian Animal Research Authority (paper I and II; permit number 2012463) and by the Norwegian Food Safety Authority (paper III; permit number 11321).

2.1.1 The three R's

Throughout the experiments reported, numerous efforts were made to adhere to the three R's: Replacement, Reduction, and Refinement (Russell & Burch, 1959).

Replacement

Replacement refers to utilizing methods that replace the use of animals with some alternative method, or replace animals considered more sentient, with animals considered less sentient. The rat has been chosen as the model animal in the experiments reported in this thesis. Hence, replacement on ethical grounds has not been considered appropriate.

Reduction

Reduction refers to utilizing methods which reduce the number of animals necessary to achieve a given amount of data. In the present experiments involving tissue collection, tissue from several brain areas and peripheral organs were collected (more than 40 samples per animal). These are stored and available for future analysis or to be shared with other scientists, lowering the need for additional experiments, which contributes to reduction.

In paper III, the hierarchical regression approach chosen for some statistical analyses (section 2.7.4) allows the targeted identification of individual factors as predictors of

selected outcomes. The hierarchical regression analysis does not allow for concluding causality but allows for generating new hypotheses, which can subsequently be tested experimentally. Such an approach requires a much smaller number of animals in total than related methods involving, e.g., machine learning and hence is in line with the R of reduction.

Refinement

Refinement refers to utilizing methods that reduce pain, stress, and suffering and increase the animals' well-being. Several measures were taken to adhere to refinement throughout the experiments performed in this thesis. In papers I and III, animals were implanted with telemetric transmitters to allow for recording of biopotentials. Once recovered from surgery, subcutaneous telemetric transmitters enable continuous undisturbed data collection in the animal's home cage for weeks and months. This contrasts with more invasive methods, which involve surgical application of a plug to the skull, which is subsequently wired to collect electrophysiological data for a limited amount of time (hours). Thus, the use of telemetric transmitters adheres to the R of refinement (un-invasive post-recovery) and the R of reduction (allows collection of more data from the same animal).

The three R's exist as ethical means to strive toward. Thus, work to adhere to the three R's is never complete. In the experiments presented in this thesis, there are several places where measure can be and are planned to be, taken to further adhere to the R of refinement. Firstly, in papers I and III animals implanted with telemetric transmitters were single-housed post-surgery due to risk of infection and improper healing of surgical wounds due to cagemate interference. To allow data to be directly comparable, time-matched controls and experimental animals not implanted with transmitters were likewise single-housed throughout the experimental protocol. Future experiments should test the impact of single- or group housing on the outcomes of the present model, which may result in no longer needing to single-house all animals, in accordance with the R of refinement.

Secondly, in the present experiments, analgesics were administered by needle injection to all rats implanted with telemetric transmitters post-surgery. Injections were performed twice each day for three days. Less invasive techniques for analgesic administration exist. The Norwegian Food Safety Authority has granted permission to administer analgesics mixed into a commercially available nut paste (Nutella). This method has been documented not to induce the release of glucocorticoids to the bloodstream and is thus considered stress-free (Abelson, Jacobsen, Sundbom, Kalliokoski, & Hau, 2012), and hence in line with the R of refinement.

2.1.2 Classification of severity

Related to the three R's is the classification of the total severity of procedures performed on each animal (Smith et al., 2018). European law requires that all experiments must be classified as either mild, moderate, severe, or terminal. The severity for all animals in the experiments presented in this thesis was categorized as either mild or moderate, the major difference being whether the animal underwent surgical implantation of a telemetric transmitter (which is automatically classified as moderate severity) or not. In papers I and III, several animals were implanted with telemetric transmitters, then underwent one three- or four-day shift work protocol as described below (section 2.2), before they were subjected to a 4-5 week washout period and subsequently commencing a three-day shift work protocol once again. Usually, it is considered most ethical to subject each animal to one experiment only, and reuse of animals in multiple experiments is generally prohibited. However, in this case, reuse was approved by the Norwegian Food Safety Administration since the implantation of the telemetric transmitter comprised the primary factor contributing to classifying the procedure as moderate severity. Whether the animal was subsequently subjected to one or two three-day exposures to simulated shift work was not sufficient to alter severity classification for the individual animal. Instead, in this specific case, the reuse of animals can be considered an ethical choice, in line with the R of reduction.

2.1.3 Choice of rat strain

Male Wistar and Sprague Dawley rats were used in the experiments. Different rat strains were chosen because the supplier (Taconic, Denmark) stopped delivering the Wistar strain in 2017, when data for paper II was being collected. Therefore, Wistar rats from a different supplier (Janvier, France) were ordered. However, upon arrival, it became apparent that these animals were behaviorally different from the Wistar rats originally supplied by Taconic. The animals from Janvier appeared less responsive to stress, with less fecal excretion upon initial handling. Moreover, they exhibited large variation in sensitivity and responsivity to the anesthetic medication administered before surgical implantation of telemetric transmitters, with some individuals requiring two injections to achieve a proper anesthetic effect. Differences between animals of the same strain and different suppliers have also been reported in the literature previously (e.g., Goepfrich, Gluch, Friemel, & Schneider, 2013; Palm, Hävermark, Meyerson, Nylander, & Roman, 2011). Therefore, the Sprague Dawley strain from Taconic was chosen rather than Wistar from Janvier. Statistical comparisons of results from the two rat strains suggest that these strains respond similarly to the experiments in paper II (see Marti et al., 2017 for details).

2.1.4 Housing

All animals were acclimatized to laboratory conditions and initially group-housed. After surgery, animals were single-housed to allow recovery of surgical wounds without disturbance from cagemates. To allow direct comparison of results, all animals were single-housed during the experimental protocol. Animals were kept on a standard LD cycle with 12 hours of lights on, starting at zeitgeber time 0 (ZT0), and 12 hours of lights off, starting at ZT12. Lights were gradually dimmed on and off across 1 hour, fully on at ZT1 and fully off at ZT13. Food and water were available *ad libitum* throughout the experiment

2.2 Simulated shift work procedure

To simulate shift work, rats were exposed to forced activity for 8 hours per day, centered either in their normal active phase (active work; ZT14–22) or in their normal rest phase (rest work; ZT2–10). The animals were placed in automatically rotating wheels revolving at 3 rotations per minute, corresponding to approximately 1.1 km of linear distance in an 8-hour session. In comparison, a rat with free access to a running wheel voluntarily travels up to 6-9 km per day (Rodnick, Reaven, Haskell, Sims, & Mondon, 1989). Thus, the pace is sufficient to keep the animals awake and active (reported in Grønli et al., 2017) but should not be considered physically exhausting. Between work sessions, animals were housed in their home cage.

2.3 Surgical procedure

Animals in papers I and III were implanted with telemetric transmitters for continuous wireless recording of body temperature and EEG and electromyography (EMG). The transmitters were placed in subcutaneous pockets in the dorsomedial lumbar region (4ET transmitters) or the neck region (F40-EET transmitters). Body temperature was monitored peripherally at the site of the transmitter. Intracranial electrodes for collection of EEG signals were placed epidurally (frontal-frontal derivation 2 mm anterior to bregma and 2 mm laterally to the midline; frontal-parietal derivation 2 mm anterior to lambda and 2 mm lateral to the midline) and secured to the skull. Two electrodes were attached to the neck muscle for the collection of EMG signals. All animals were allowed at least 14 days of recovery post-surgery before entering the experiment (Moscardo & Rostello, 2010).

2.4 Collection of telemetric transmitter data

Telemetry signals were collected through receivers placed directly beneath the animals' home cage or next to the rotating wheel during simulated shift work. Body temperature was sampled at 50 Hz, and data were stored per 10 seconds. Data from biopotentials (EEG and EMG) were recorded at a sampling rate of 250 Hz.

2.5 Paper I

The experiments in paper I were designed to investigate the immediate effects of simulated night shift work and the associated shift in food intake on metabolic regulation. Telemetric transmitters were implanted, and data recorded, as described in sections 2.3 and 2.4. The rats (n=40) underwent 4 days of undisturbed baseline recording before commencing the shift-work protocol for 4 consecutive days (active workers n=16, rest workers n=24), followed by 8 days of recovery in LD conditions (active workers n=11, rest workers n=14). Food intake was monitored during baseline and simulated shift work. Body weight and body temperature were monitored throughout the experiment.

After a minimum of five weeks washout, the animals were again subjected to the shift work protocol for 3 days. After the last shift, animals were fasted for 2 hours (to avoid the immediate effects of food intake on liver gene expression) and subsequently sacrificed for liver tissue collection. A separate group of animals never exposed to shift work was sacrificed at the same time as time-matched controls (ZT0 or ZT12, n=10).

2.5.1 Analysis of food intake and body weight

Food intake was used as a measure for energy intake and body weight as a measure for overall energy balance. During baseline and simulated shift work, measures were performed to estimate energy balance at 8-hour and 16-hour intervals (corresponding to before and after the 8-hour work shift). During recovery, to avoid unnecessary disturbance, animals were weighed every four days only.

2.5.2 Analysis of body temperature data

Body temperature data from telemetric transmitters were used as a measure for energy expenditure (Abreu-Vieira, Xiao, Gavrilova, & Reitman, 2015; Vinales, Begaye, Thearle, Krakoff, & Piaggi, 2019). 24-hour mean, 12-hour rest phase mean (lights on; ZT12-24), and 12-hour active phase mean (lights off; ZT0-12) relative to baseline were calculated.

2.5.3 Analysis of liver gene expression

Liver tissue was analyzed using the real-time polymerase-chain-reaction (PCR) technique from animals sacrificed after three days of simulated shift work and their time-matched controls. This method, requiring only a small amount of tissue, amplifies the amount of RNA in each sample and subsequently allows the measurement of the relative amount of specific RNAs transcribed in the tissue at the time of sacrifice. The relative expression of gene transcripts involved in important hepatic metabolic pathways, including lipid, glucose, and protein metabolism were measured. See Table 1 for overview.

Table 1.

Abbreviations, full names, and main functions of hepatic transcripts analyzed in paper I.

Abbreviation	Full name	Main function
FAS	Fatty acid synthase	Fatty acid synthesis
SCD1	Stearoyl-CoA-desaturase	Fatty acid desaturation
DGAT1	Diacylglycerol O-acyltransferase 1	Triglyceride synthesis
DGAT2	Diacylglycerol O-acyltransferase 2	Triglyceride synthesis
GPAM	Glycerol-3-phosphate acyltransferase 1, mitochondrial	Triglyceride synthesis
SREBP1c	Sterol regulatory element-binding protein 1c	Fatty acid/triglyceride synthesis
PPARα	Peroxisome proliferator-activated receptor alpha	Fatty acid oxidation
PPARγ	Peroxisome proliferator-activated receptor gamma	Adipocyte differentiation
IRS2	Insulin receptor substrate 2	Mediation of insulin effects
PYGL	Phosphorylase, glycogen, liver	Glycogen breakdown
mTOR	Mechanistic target of rapamycin	Mediation of cellular metabolic stress response, protein synthesis
ChREBP	Carbohydrate responsive element binding protein	Triglyceride synthesis in response to carbohydrate

2.5.4 Statistics

Baseline body weight change, food intake, and mean body temperature were compared between groups using student's unpaired t-test. Linear mixed model analysis was used to assess body weight changes, food intake, and mean body temperature across workdays and between groups. This method allows for analysis of repeated measures with missing data points. Differences in gene expression (fold-change) between groups were evaluated using Student's unpaired t-test. Statistical significance was accepted at $p<.05$. Where statistically significant effects were observed, pairwise comparisons were performed.

2.6 Paper II

The experiment in paper II was designed to examine the effects of 3 days of simulated shift work on brain protein synthesis markers. Rats were exposed to the simulated shift work protocol for 3 consecutive days (active workers n=10, rest workers n=10), placed in their home cage for 2 hours, and subsequently sacrificed for brain tissue collection. Time-matched controls were sacrificed at the same time (ZT0 or ZT12, n=20).

2.6.1 Cap-pulldown and western blot analysis

Bilateral hippocampus and PFC samples were analyzed using the cap-pulldown method. In this procedure, the homogenized sample is incubated with m^7GTP-agarose beads, which resemble the mRNA 5' "cap". Subsequently, unbound protein is washed away. Therefore, this method allows the quantification of the relative amounts of protein that is actively bound to the mRNA cap, rather than the total available protein in the sample (from here referred to as input samples, or inputs).

Protein samples from the cap-pulldown assay as well as input samples, were analyzed using the western blot technique. Sample-containing nitrocellulose membranes were probed with antibodies specific to proteins (total and phosphorylated) involved in promotion and repression of translation initiation, as well as related regulatory proteins. See Table 2 for overview. S6K1 and Arc were analyzed in inputs only, as

these proteins do not interact directly with the cap. Lastly, relative protein expression was quantified using densitometric analysis. Input samples were normalized to GAPDH and cap-pulldown samples normalized to total eIF4E on the same lane. Phospho-protein was normalized to total protein on the same lane.

Table 2.

Abbreviations, full names, and main functions of proteins analyzed in paper II.

Abbreviation	Full name	Main function
(p-)eIF4E	(Phosphorylated) eukaryotic initiation factor 4E	Translational promoter
eIF4G	Eukaryotic initiation factor 4G	Translational promoter
(p-)BMAL1	(Phosphorylated) brain-and-muscle arnt-like protein 1	Translational promoter
(p-)pS6K1	(Phosphorylated) ribosomal protein S6 kinase beta-1	Phosphorylates BMAL1
4E-BP2	eIF4E-binding protein 2	Translational repressor
FMRP	Fragile X mental retardation protein	Translational repressor
CYFIP1	Cytoplasmic FMRP-interacting protein 1	Translational repressor
Arc	Activity-regulated cytoskeleton-associated protein	Synaptic plasticity regulator

2.6.2 Statistics

Differences between time-points, work conditions, and brain regions were statistically analyzed using 2x2 factorial analysis of covariance (ANCOVA). Fisher's LSD was applied as a post-hoc test. Statistical significance was accepted at $p<.05$. Cohen's d was calculated as a measure of effect size. When interpreting Cohen's d, 0.2 is considered small, 0.5 medium, and 0.8 large effect size, respectively (Cohen, 1988).

2.7 Paper III

The experiments in paper III were designed to examine the effects of simulated shift work on spatial memory performance on the Morris Water Maze (MWM) task and

how individual differences in daily rhythmicity, sleep drive, and serum corticosterone predict spatial memory performance and protein markers of PFC functioning after simulated shift work. Rats implanted with telemetric transmitters first underwent 5 days of baseline recording. Then, the animals received 3 days of training in the MWM before commencing shift-work protocol for 3 consecutive days (active workers n=16, rest workers n=24). Immediately after the third shift, recall on the MWM was tested. After at least four weeks washout, animals once again underwent 5 days of baseline recording, followed by the shift work protocol for 3 consecutive days. Two hours after the last shift, animals were sacrificed for blood and brain tissue collection. Time-matched controls were sacrificed at the same time (ZT0 or ZT12, n=16).

2.7.1 Morris Water Maze Task

In the MWM task (Morris, 1984) the animal is placed in a pool filled with water and required to swim to locate a platform hidden beneath the water surface. This task was chosen for two main reasons. Firstly, it is fast to administer, thus allowing the testing of several animals across a short period of time and providing flexibility when combined with other experimental protocols. Secondly, the MWM task does not many involve food reward and hence does not require food deprivation to motivate the animals to perform. In the MWM task, the animal is inherently motivated to escape the water, allowing the maintenance of *ad libitum* access to food, in accordance with Refinement and lessening experimental variability.

Prior to simulated shift work, all animals were trained on the MWM task. Training occurred across 3 days. Each animal had 4 trials per day, starting at approximately ZT6. Training and testing were performed by the same experimenter under dim red-light conditions. On the first day, the platform was located in the middle of the pool, visible above the water surface. On the next two days, the platform was located in one specific quadrant of the pool, hidden below the water surface. Black paint was added to the water to prevent the rats from seeing the platform. Immediately after the third work shift, the animals' recall of the platform location was tested (rest workers

at ZT10, active workers at ZT22). Latency to platform and swim speed were calculated for each animal.

2.7.2 Analysis of telemetric transmitter data

Telemetric transmitters were implanted, and data recorded, as described in sections 2.3 and 2.4. Telemetric transmitter recordings were used to generate data regarding daily rhythm dynamics and sleep drive.

Daily rhythm dynamics

To estimate changes and individual variations in daily rhythm dynamics, changes in the body temperature rhythm amplitude and REM sleep latency after the second work shift were measured. Body temperature rhythm was analyzed with cosinor analysis, performed on 72-hour baseline data and 72-hour work data. Rhythm amplitude change from baseline was calculated by subtraction. Wakefulness, non-REM (NREM) sleep, and REM sleep were manually scored based on EEG and EMG signals, following the criteria by Neckelmann and Ursin (1993). REM sleep latency was defined as the latency to 3 consecutive 10-second epochs of REM sleep.

Sleep drive

To estimate changes and individual variations in sleep drive, the average duration of NREM sleep bouts between work shifts and the amount of slow-wave energy (SWE) in quiet wakefulness during work shifts were measured. A NREM sleep bout was defined as 3 or more consecutive 10-second epochs of NREM sleep. Quiet wakefulness was distinguished from active wakefulness based on the EMG amplitude in each epoch relative to the EMG amplitude in all other epochs. In epochs where EMG amplitude was below or equal to the 33rd percentile amplitude, wakefulness was characterized as quiet wakefulness (Grønli, Rempe, Clegern, Schmidt, & Wisor, 2016). EEG slow-wave activity (SWA; average spectral power in the 1-4 Hz range) was characterized using Fast Fourier Transform analysis. The accumulation of SWA over time, slow-wave energy (SWE), in quiet wakefulness was calculated.

2.7.3 Serum and tissue analysis

PFC tissue was collected, and expression of translational promoters (i.e., eIF4E, BMAL1, and S6K1) and related regulators of synaptic plasticity (i.e., Arc) were analyzed using cap-pulldown and western blot as described in section 2.6.1.

Serum corticosterone analysis

Serum corticosterone was analyzed with the enzyme-linked immunosorbent assay (ELISA) technique according to the manufacturer's instructions. The kit was selected based on previous data from the present laboratory showing high correlation to other kits in addition to high detection of corticosterone even at low concentrations (Rød, Harkestad, Jellestad, & Murison, 2017).

2.7.4 Statistics

Group differences were analyzed using Student's unpaired t-test. Differences in parameters following work relative to baseline were calculated using Student's paired t-test. Statistical significance was accepted at $p<.05$. Cohen's d was calculated as a measure of effect size (see section 2.6.2).

Hierarchical regression

To determine predictors of spatial performance and PFC protein expression, we used the multiple regression analysis with the hierarchical regression approach. This approach allows testing of whether certain variables can predict part of the variation in a second variable, and whether adding more variables can enhance this prediction, after controlling for the increased number of variables (indicated by the adjusted R^2)(Greenland, 1994).

Several models were tested, most notably the prediction of (1) work condition, (2) daily rhythm dynamics, including body temperature rhythm amplitude change and REM sleep latency, (3) markers of sleep drive, including average length of NREM sleep bouts and SWE in quiet wakefulness, and (4) serum corticosterone, on variation of spatial performance and PFC protein expression data.

3. Summary of results

For full results, see the results sections of paper I, II, and III.

3.1 Paper I

In paper I, the effects of 3-4 consecutive days of simulated shift work on markers of energy balance and liver gene expression were investigated.

3.1.1 Simulated night shift work shifts the timing of food intake

Both active and rest workers reduced their food intake, relative to baseline (Figure 4A). In addition, rest workers shifted the timing of food intake to the rest phase. This shift stabilized on the third workday (Figure 4B).

Both active and rest workers lost body weight throughout the simulated shift work protocol, although rest workers lost more. Consequently, both groups gained weight during the 8-day recovery period, but the rest workers gained more. At the end of the recovery period, active workers had regained body weight to baseline levels while rest workers remained slightly below baseline.

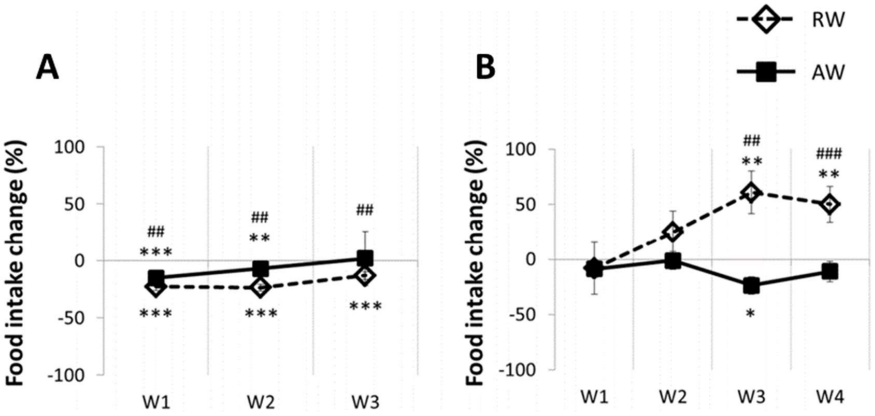

Figure 4. Food intake across one shift work period for active workers (AW) and rest workers (RW): (A) 24 h food intake; (B) 8 h food intake (during work). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1–4 indicates workdays 1 to 4. W4 not included in 24 h food intake due to missing measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to baseline. ## $p < 0.01$; ### $p < 0.001$, between groups. Figure modified from paper I (Marti et al., 2016), with permission.

3.1.2 Simulated night shift work alters mean body temperature

Both active and rest workers exhibited elevated 12-hour mean body temperature associated with work-sessions, compared to baseline (Figure 5A-B). Mean body temperature levels were initially returned to baseline levels on recovery day 2 in both groups. However, rest workers unexpectedly developed progressive hypothermia in the rest phase (Figure 5B). Measurements were stopped after eight days. Hence, it is unclear how long this effect lasted.

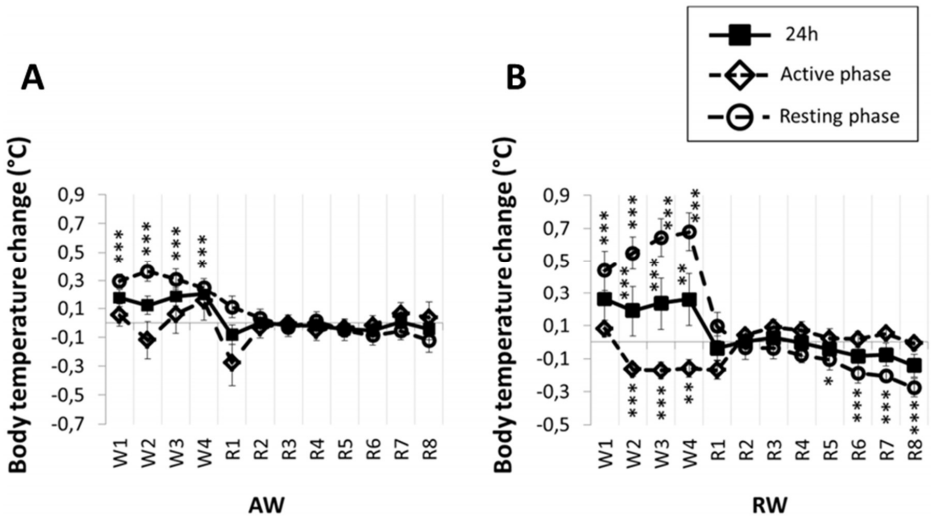

Figure 5. Mean body temperature during one shift work period (workday 1-4, W1–4) and recovery (recovery day 1-8, R1–8) for (A) active workers (AW) and (B) rest workers (RW). Data are shown as mean percentage change relative to baseline. Error bars indicate SEM. ** $p < 0.01$; *** $p < 0.001$, compared to baseline. Figure modified from paper I (Marti et al., 2016), with permission.

3.1.3 Simulated night shift work alters liver markers of metabolism

No transcripts were selectively up or downregulated in active workers, relative to their time-matched controls. In contrast, rest workers exhibited upregulation of *IRS2* involved in regulating insulin response, and upregulation of *GPAM*, involved in lipid synthesis, relative to time-matched controls (Figure 6). *PPARα*, involved in lipid oxidation, was downregulated in rest workers relative to time-matched controls. Overall, these transcriptional changes seem to reflect alterations in the hepatic response to simulated shift work, promoting the storage of lipids and glucose dysregulation, to the most considerable extent in rest workers.

Taken together, the results of paper I show that 3-4 consecutive days of simulated night shift work are sufficient to induce acute changes to metabolic regulation, reflected in changes in markers of energy balance and hepatic gene expression, some of which take longer than 8 days to recover.

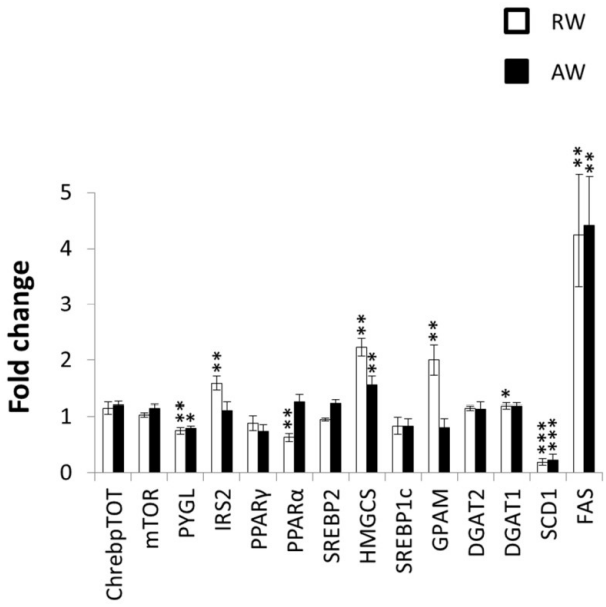

Figure 6. Expression levels of key metabolic genes in the liver from rest workers (RW; sacrificed at zeitgeber time 12, ZT12) and active workers (AW; sacrificed at ZT0) following three work sessions, compared to time-matched controls (RW controls sacrificed at ZT12; AW controls sacrificed at ZT0). Fold changes for each gene represent the relative difference between active and rest workers and their time-matched controls. Error bars indicate SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. For full gene names, see table 1. Figure reprinted from paper I (Marti et al., 2016), with permission.

3.2 Paper II

In paper II, the effect of simulated shift work on markers of cap-dependent translation initiation in the hippocampus and PFC, both important for cognitive functioning, were investigated.

3.2.1 Simulated night shift work disrupts BMAL1-driven translation regulation in the PFC

Data from time-matched controls suggested that translation initiation was being promoted in the PFC at ZT12, at the end of the rest phase. In the hippocampus, no statistically significant time-of-day variations were observed.

Following rest work, the cap-bound translational promoter p-BMAL, its regulator p-S6K1, and the synaptic plasticity regulator Arc were all downregulated in the PFC, relative to time-matched controls (Figure 7A, 7C, 7G). There were no significant changes to any of the examined proteins in the hippocampus of rest workers, or any of the examined proteins in active workers, either in the PFC or the hippocampus (Figure 7), relative to time-matched controls.

Taken together, the results of paper II suggest that simulated night shift work impairs cap-dependent translation initiation in the PFC at a time-point when the translational machinery is normally being promoted (illustrated in Figure 7I).

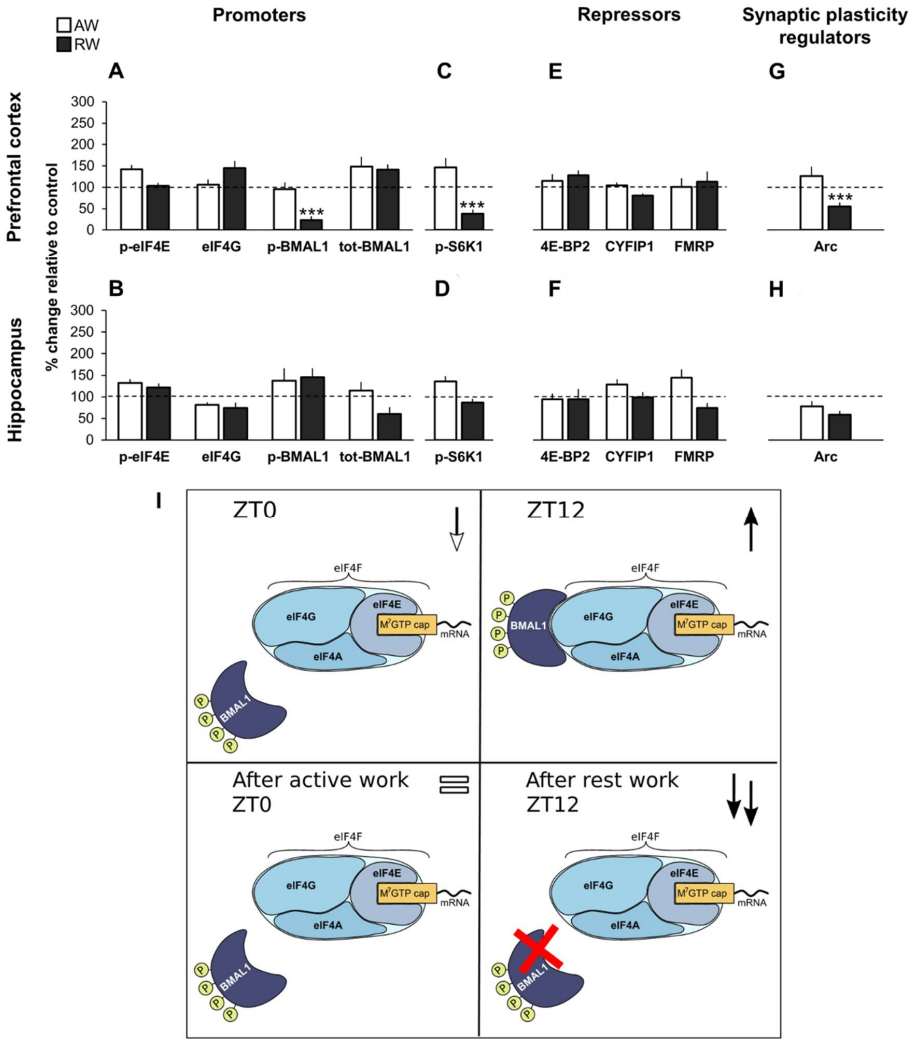

Figure 7. Effect of simulated shift work on promoters and repressors of cap-dependent translation initiation and synaptic plasticity regulators, relative to time-matched undisturbed controls. (A, E) m^7GTP pull-down analysis of PFC and (B, F) hippocampus lysates. (C, G) Western blot analysis of PFC and (D, H) hippocampus lysates. Rats were exposed to forced activity during the active phase (AW, active work; brain tissue was collected at zeitgeber time 0, ZT0) or during the rest phase (RW, rest work; brain tissue was collected at ZT12). Quantification of immunoblot is expressed as percentage change relative to time-matched undisturbed control (normalized to 100%). Error bars represent SEM. *** $p < 0.001$. (I) Schematic model of promotion of translation initiation in undisturbed animals (ZT0 and ZT12) and following simulated shift work (active work and rest work). ↓ decreased expression compared to ZT12; ↑ increased expression compared to ZT0; = similar expression compared to ZT0; ↑↑ increased expression compared to ZT12. For full protein names, see table 2. Figure reprinted from paper II (Marti et al., 2017), with permission.

3.3 Paper III

In paper III, the effects of simulated shift work on spatial memory performance, and the prediction of individual variation in daily rhythmicity, sleep drive, and serum corticosterone in response to simulated shift work, on spatial memory performance and protein markers of PFC functioning after simulated shift work, were investigated

3.3.1 Simulated night shift work impairs spatial memory performance

Before entering the shift work protocol, rest workers and active workers performed similarly on the MWM task (Figure 8A). Following simulated shift work, rest workers required longer time on average to locate the hidden platform, relative to their pre-work training session (Figure 8A) and compared to active workers (Figure 8B). However, considerable individual differences were observed, and some rest workers performed on par with active workers.

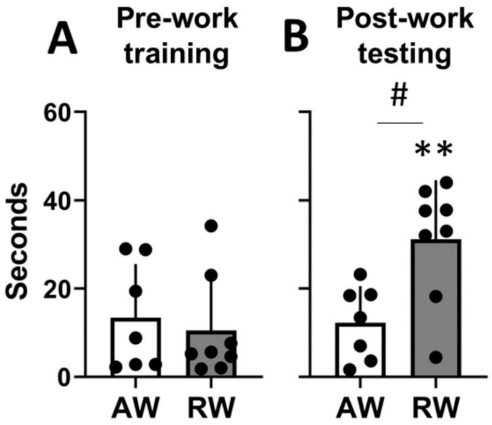

Figure 8. Latency to platform on the Morris Water Maze task, for active workers (AW) and rest workers (RW). (A) The last training trial before commencing the 3-day work period. Training occurred around zeitgeber time 6, ZT6. (B) The first testing trial immediately after the third work shift, AW at ZT20, RW at ZT10. Plots show mean ± SEM, with scatter plot overlaid. N = 7–8/group. ** $p < 0.01$, compared to pre-work training. # $p < 0.05$, between groups. Figure reprinted from paper III (Marti et al., 2020), with permission.

3.3.2 Simulated night shift work alters markers of daily rhythmicity and sleep drive

Following the 3-day shift work protocol, rest workers exhibited reduced circadian rhythm amplitude relative to baseline. Moreover, REM sleep latency following the second work session was reduced in rest workers compared to active workers. The average length of NREM sleep bouts was similar in both groups. However, rest workers exhibited a higher accumulation of SWE in quiet wakefulness, as described previously (Grønli et al., 2017), without change to time spent in sleep. Importantly, considerable individual variation in all these parameters was observed, with substantial overlap between the two groups.

3.3.3 Type of work, markers of daily rhythmicity, and sleep drive predict spatial memory performance

Multiple regression analysis with a hierarchical regression approach was used to test the predictive value of different rhythm and sleep-related markers on spatial memory performance.

First, a simple model with work condition as the only predictor of latency to platform in the MWM task was tested. Work condition alone modestly predicted MWM performance ($R^2 = 0.23$). Next, daily rhythm parameters, change in body temperature rhythm amplitude and REM sleep latency, were added as predictors. These markers of daily rhythmicity slightly enhanced the ability of the model to predict MWM performance (adjusted R^2 increased by 0.08). Lastly, markers of sleep drive, NREM sleep bout duration, and cumulated SWE in quiet wakefulness were added to test if these markers further predicted MWM performance. The addition of sleep drive markers drastically increased the prediction of the model (adjusted R^2 increased by 0.27).

Taken together, these results suggest that all three predictors, work condition, markers of daily rhythmicity, and markers of sleep drive, contribute to predict the individual variation in spatial memory performance following simulated shift work.

3.3.4 Type of work, markers of daily rhythm dynamics, and sleep drive predict different aspects of PFC translational regulators

In paper III the results of paper II, showing impairment to the cap-dependent translational machinery following rest work, were replicated. Expression of the cap-bound translational promoter p-BMAL1 and the synaptic plasticity regulator Arc were reduced in the PFC following rest work ($p<.05$), with large effect sizes for reduced p-eIF4E and the p-BMAL1 regulator p-S6K1, relative to time-matched controls. Individual variation was also observed in these parameters.

To evaluate whether work condition, parameters related to daily rhythmicity, and sleep drive predicted the cortical expression of translational promoters and related regulators, multiple regression analysis with hierarchical regression approach was once again conducted. Work condition strongly predicted expression of p-S6K1 (R^2=0.87). Adding daily rhythm parameters enhanced predictive power of expression of cap-bound p-eIF4E and p-BMAL1 (adjusted R^2 increased by 0.10 and 0.11, respectively). Markers of sleep drive slightly enhanced prediction of expression of cap-bound p-eIF4E and p-S6K1 (adjusted R^2 increased by 0.03 and 0.02, respectively).

The expression of the synaptic plasticity regulator Arc was only predicted by work condition, not by any of the other tested variables (negative adjusted R^2 suggesting no fit of any of the models).

3.3.5 Serum corticosterone predicts different aspects of PFC translational regulators.

At the group level, serum corticosterone was significantly increased in active workers relative to time-matched undisturbed controls, and significantly reduced following rest work, relative to time-matched controls (Figure 9). As with other variables, considerable individual variations were observed. Thus, the ability of serum corticosterone to increase the prediction of the above-described model, predicting markers of cap-dependent translation initiation, was tested.

Adding serum corticosterone as a predictor variable enhanced prediction of cap-bound p-eIF4E (adjusted R^2 increased by 0.57) and slightly enhanced prediction of cap-bound p-BMAL1 and p-S6K1 (adjusted R^2 increased by 0.01 and 0.02, respectively). Arc was not predicted by serum corticosterone (negative adjusted R^2).

Taken together, the results of paper III suggest that the four predictors, work condition, markers of daily rhythmicity, markers of sleep drive, and serum corticosterone, predict different aspects of spatial memory performance and PFC translational regulators following simulated shift work.

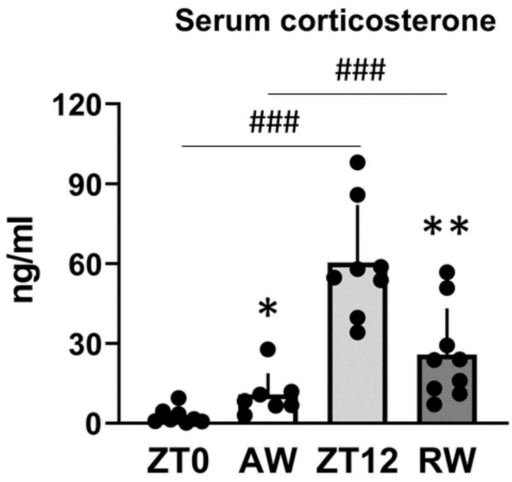

Figure 9. Concentration of serum corticosterone in undisturbed controls (zeitgeber time, ZT0 and ZT12) and following 3 days of active work (AW, ZT0) and rest work (RW, ZT12). Plots show mean ± SEM, with scatter plot overlaid. N = 7–9/group. * $p < 0.05$, ** $p < 0.01$, compared to time-matched controls. ### $p < 0.001$, between groups. Figure reprinted from paper III (Marti et al., 2020), with permission.

4. Discussion

The present work aimed to investigate the acute effects of simulated night shift work on metabolic and brain functioning and to understand how individual factors may predict brain functioning following simulated shift work.

The findings suggest that just 3-4 days of simulated night shift work alters metabolic regulation and markers of brain functioning. Moreover, type of work, as well as individual variation in daily rhythm parameters, markers of sleep drive, and serum corticosterone predicted different aspects of cognitive performance and markers of brain functioning following simulated shift work.

The findings of papers I, II, and III have been discussed in detail within the respective papers. In this section, some of these findings will first be briefly reappraised individually, followed by a more general discussion to link findings together as well as place the findings in a broader research context. The potential impact on human shift workers and recommendations for future research will be presented. Lastly, the validity of the model will be critically evaluated before the final concluding remarks.

4.1 Effects of simulated shift work

4.1.1 Metabolism

Metabolic effects of simulated shift work have been the primary area of study for animal models and are perhaps the best-understood aspect of health effects of shift work. There is reason to assume that a shift in the timing of food intake contributes to the negative health effects of shift work (Banks et al., 2015; Salgado-Delgado, Angeles-Castellanos, et al., 2010; Skene et al., 2018). The results of paper I show that changes in markers of liver functioning, as well as dysregulation of energy balance, occur not only in the long term (Gan et al., 2015; Gao et al., 2020; Rivera et al., 2020) but also in the short term. Following 4 days of simulated night shift work, rats exhibited rest phase hypothermia which declined progressively throughout the 8-day recovery protocol. This suggests a positive energy balance resulting from simulated night shift work and, presumably, shifted timing of food intake. The effect can be

considered compensatory since similar rest phase hypothermia has been observed after prolonged exposure to semi-starvation in rats (Siyamak & Macdonald, 1992). Similarly, an immediate reduction in energy expenditure during daytime sleep has been observed in a study of simulated night shift work in human participants under strictly controlled conditions (McHill et al., 2014). Such a reduction in energy expenditure, resulting in a net positive energy balance, is associated with weight gain. Night shift work is associated with an increased risk of obesity, which in turn is associated with disease and metabolic dysregulation, in both humans and animal models (Pedersen et al., 2012; Rivera et al., 2020; Salgado-Delgado et al., 2008; Salgado-Delgado et al., 2013).

Metabolic changes in response to simulated night shift work relate not only to energy balance but also to circulating markers of metabolic regulation. One study in rats reported dysregulation of circulating lipids after five weeks of simulated night shift work, associated with eating during the normal rest phase (Salgado-Delgado et al., 2008). A recent study in humans showed that after just 4 days of eating at night, levels of circulating lipids after a meal reached the same level as during the day, but requiring only half the amount of calories to reach this level (Grant, Czeisler, Lockley, & Rahman, 2020). The present finding of increased hepatic expression of genes associated with lipid synthesis (*FASN*, *GPAM*, and *PPARα*) after just three days of simulated night shift work is in line with this recent finding, supporting the notion that a rapid change in metabolic regulation may occur following night shift work.

4.1.2 Cognition and brain function

The findings of paper II show that the BMAL1-driven translational machinery is impaired within the PFC after only three days of simulated night shift work, at a time-point where this machinery is normally active. Some of these findings, including reduced cap-binding of BMAL1 after simulated night shift work, were also replicated in paper III.

Recent work suggests that there exists a reciprocal relationship between cytoplasmic BMAL1 and mTOR. mTOR integrates intra- and extracellular signals to regulate several processes, including cap-dependent protein synthesis, in both central and peripheral tissues (Laplante & Sabatini, 2009). BMAL1 is well-known as a clock gene, regulating cellular rhythms within the cell's nucleus (King & Takahashi, 2000; Mohawk et al., 2012). Its role in the cell's cytoplasm has been less clear. However, in light of scientific discoveries in the last 6-7 years, mTOR stands out as a key regulator of cytoplasmic BMAL1 (Lipton et al., 2017; Lipton et al., 2015). It was recently shown that mTOR activity modulates circadian rhythm period and rhythm amplitude in cell cultures (Ramanathan et al., 2018). Overactivation of mTOR in vivo also alters the circadian rhythm period in mice (Cao et al., 2013). Results from a mathematical modeling approach suggest that mTOR regulates rhythmicity by (1) exerting translational control on BMAL1, and (2) controlling the post-translational localization of BMAL1 (Guerrero-Morin & Santillan, 2020).

However, the relationship between mTOR and clock mechanisms goes both ways. mTOR activity is itself under circadian clock control (see Cao, 2018 for review). Work in mice has shown that mTOR expression oscillates in a circadian fashion both within the hippocampus and the frontal cortex (Khapre et al., 2014; Saraf et al., 2014). However, the clock mechanisms that drive mTOR rhythmicity in different tissues are still unclear. One study suggests that BMAL1 is capable of regulating mTOR rhythmic activity in both cell culture and different bodily tissues, including the frontal cortex (Khapre et al., 2014). Thus, mTOR not only regulates BMAL1 translational activity within the PFC, but BMAL1 may also itself regulate mTOR rhythmicity within the same brain region. The mechanisms of this potential link remain to be elucidated.

The findings of paper III show that simulated night shift work is associated with disturbed cognitive performance. Although the results of paper II showed that the BMAL1-driven translational machinery and related synaptic plasticity markers were impaired in the brain following simulated night shift work, it was unclear whether the simulated shift work protocol was sufficient to induce impairments in cognitive

performance. Thus, the results of paper III demonstrated that the simulated night shift work protocol was indeed sufficient to impair spatial memory performance on the MWM task. However, the MWM task is considered to be primarily hippocampus-dependent (Brandeis et al., 1989; Tucker, Velosky, & McCabe, 2018), but the results of paper II showed no significant alterations to the BMAL1-driven translational machinery within this region.

It is conceivable that the effects of simulated shift work were so dramatic that all cognitive domains suffered, but this explanation seems unlikely considering that a previous study in rats showed unaltered cognitive performance on an instrumental task after five weeks of simulated night shift work (Leenaars et al., 2012). Rather, it is possible that the procedure indeed induced functional deficits within the hippocampus, but that these were not detected in paper II since analyses were run on whole-hippocampus lysates. Previous work has shown that the hippocampus displays robust rhythmicity in LTP induction, indicative of rhythmicity in synaptic plasticity and protein synthesis, but these rhythms are not uniform across different compartments of the hippocampus (see Gerstner & Yin, 2010 for review). LTP induction has been shown to peak during the dark phase within the dentate gyrus, and during the light phase in the CA1 region of the rat hippocampus (Harris & Teyler, 1983). Thus, any potential sub-region-specific effects of simulated shift work within the hippocampus cannot be ruled out.

Results from the hierarchical regression analysis performed in paper III showed that different aspects of the work condition, as well as individual variation in daily rhythm dynamics, sleep drive, and serum corticosterone predicted different aspects of cognitive performance and markers of brain functioning after simulated shift work. The findings will be discussed further in later sections. However, the expression of the synaptic plasticity regulator Arc was notably not predicted by any of the tested factors. Arc is central to the regulation of multiple aspects of synaptic plasticity (Nikolaienko et al., 2018), and modulated by sleep and wake (Grønli et al., 2014). Of the predictors that were tested in paper III, Arc has primarily been studied in the context of sleep and appears to be tightly regulated by time spent in sleep (see paper

II for discussion). It is unclear whether Arc is modulated by changes in homeostatic sleep drive per se, although enhanced slow-wave activity during sleep and Arc protein expression within the frontal cortex have been observed in rats trained on a motor task (Hanlon, Faraguna, Vyazovskiy, Tononi, & Cirelli, 2009). Few studies have examined circadian variations in Arc mRNA or protein, although Arc has been implicated in the regulation of circadian plasticity within the SCN (Nishimura, Yamagata, Sugiura, & Okamura, 2003). Importantly, in the present work Arc protein expression was reduced in the PFC after simulated night shift work compared to time-matched controls in both paper II and paper III. However, the impact of this altered expression on brain functioning and cognitive performance remains to be elucidated. Novel functions of Arc are still being discovered (Ashley et al., 2018; Pastuzyn et al., 2018), and its role in conditions of circadian misalignment should be the focus of future research.

4.2 Identifying the biological mechanisms underlying the adverse health effects of shift work

So far, in papers I, II, and III, the processes of the metabolic system and the brain and synaptic plasticity processes have been considered largely separate. However, these systems do not exist in isolation. They are closely intertwined by numerous processes, and (at least in part) under the control of the central clock in the SCN. In this section, the results are considered in a broader context. Firstly, which processes may explain the findings, both relating to metabolic and brain functioning?

4.2.1 What drives the effects observed in the present experiments?

Timing of food intake

In the rat model of shift work used in this thesis, only one major variable was changed directly; the timing of activity. Lights were kept constant (12 hours on/12 hours off), ensuring that the photic entrainment cues to the master clock of the SCN remained unchanged. Altering the timing of activity indirectly altered the timing of food intake, as seen in paper I.

The link between altered feeding rhythms and metabolic disturbance is relatively well-documented. Change in the timing of food intake alters the circadian rhythmicity of metabolic organs and processes, desynchronizing them from the central clock of the SCN (Atger et al., 2015; Damiola et al., 2000; Satoh et al., 2006; Stokkan et al., 2001; Vollmers et al., 2009). Simulated shift work studies in both humans and rats suggest that this transient state of internal desynchrony causes dysregulation of blood glucose regulation, blood lipid profile, energy balance, as well as disturbing the functioning of metabolic organs, such as the liver (Banks et al., 2015; Grant et al., 2017; McHill et al., 2014; Salgado-Delgado et al., 2008; Salgado-Delgado, Nadia, Angeles-Castellanos, Buijs, & Escobar, 2010; Salgado-Delgado et al., 2013; Skene et al., 2018). Is it thus possible that the altered timing of food intake also might explain some of the *cognitive* consequences of night shift work?

The majority of work on the relationship between nutrition and brain function appears to be concerned with the effects of specific nutritional components on brain functioning (Gomez-Pinilla, 2008). Another line of research that has gained some interest in recent years concerns the effects of intermittent fasting on brain function. However, the effects of intermittent fasting are likely due to reduced total caloric intake, without necessarily impacting on the diurnal rhythmicity of food intake (Mattson, Moehl, Ghena, Schmaedick, & Cheng, 2018). Little work has examined the link between the *timing* of food intake, or the misalignment of feeding rhythms, and brain functioning.

The brain is a highly metabolically active organ, consuming about 20% of the body's total daily energy requirements (Frayn, 2010). Unlike the liver and several other metabolic organs, the brain cannot store energy and thus requires a constant stream of (primarily) glucose to maintain normal functioning (Mergenthaler, Lindauer, Dienel, & Meisel, 2013). Indeed, the brain is important for the regulation of metabolism, but functional regulation of metabolism is also crucial for brain functioning. One recent study showed that a high-fat diet affects rhythmically oscillating metabolites both within the PFC and the SCN (Tognini et al 2020). The potential for studies linking metabolic rhythms to brain functioning is thus clearly present.

It has been shown that rhythmic activity of metabolic organs does indeed impact brain functions. A study showed that restricting food availability to the rest phase in rats altered rhythmicity in hypothalamic regions involved in the regulation of sleep and arousal, in addition to regions involved in regulating energy balance (Ramirez-Plascencia, Saderi, Escobar, & Salgado-Delgado, 2017). Peripheral functions of BMAL1 in muscle have also been linked to the regulation of homeostatic aspects of sleep (Ehlen et al., 2017). Still, there does not appear to be any work studying peripheral clock functions in relation to synaptic plasticity or similar processes. Relatively simple experiments could involve restricting rats' food availability to the light phase and subsequently test cognitive performance in e.g. the MWM task or other markers of brain function such as LTP induction or cap-dependent translation initiation in different brain areas. Accordingly, future studies should examine such direct effects of peripheral metabolic rhythmicity on cognitive performance, brain functioning, and synaptic plasticity.

Stress and glucocorticoids

The release of glucocorticoids by the HPA-axis impacts several physiological systems and thus serve as a prime candidate to explain some of the negative effects of shift work at the mechanism level. The results of paper III show that serum glucocorticoid levels differed between rats exposed to simulated shift work, relative to their time-matched controls. Moreover, the level of serum glucocorticoid predicted a substantial amount of the expression of the translational promoter cap-bound p-eIF4E in the PFC. The impact of glucocorticoids on health has been studied extensively. The HPA-axis regulates responses to stress, as well as the daily shift from rest to activity (Balsalobre et al., 2000). Studies have demonstrated the impact of shift work on glucocorticoid regulation, both in animal models (Salgado-Delgado et al., 2008), human models (Caufriez et al., 2002; Goichot et al., 1998), and field studies (Harris et al., 2010). After 5 weeks of simulated night shift work, rats exhibited two peaks of glucocorticoid release; one coinciding with the start of the active phase as normal, and one coinciding with the onset of simulated night shift work (Salgado-Delgado et al., 2008). Human studies suggest that responses to simulated night shift work are acute and detectable within one circadian cycle

(Caufriez et al., 2002; Goichot et al., 1998). The present data suggest an impact of simulated shift work on glucocorticoid release, but given only one time-point for measurement, the data are difficult to interpret. The results could reflect a shift in the rhythm of glucocorticoid release, changes to the overall level of glucocorticoids, or both. Nevertheless, considering the literature as a whole, shift work appears to robustly alter glucocorticoid release, with potential consequences for both metabolic and brain functioning.

The bodily systems regulating stress and circadian rhythms are tightly intertwined. Glucocorticoids are not merely clock outputs; they are mediators through which the circadian system controls and sets rhythms throughout central and peripheral tissues (Balsalobre et al., 2000; Kinlein & Karatsoreos, 2020; Koch, Leinweber, Drengberg, Blaum, & Oster, 2017). It has been suggested that some types of stressors, like social defeat, can phase shift the locomotor activity rhythm, but this has been observed primarily in hamsters and not in rats (Tahara, Aoyama, & Shibata, 2017). However, several clock genes, including *Per*, have been shown to contain glucocorticoid response elements, and glucocorticoid receptor binding has been shown to directly impact on the regulation of these (Reddy et al., 2007; So et al., 2009). A recent study showed that glucocorticoid release regulates rhythmicity of clock genes in the cerebellum of the brain (Bering, Hertz, & Rath, 2020). Likewise, disturbance of circadian rhythmicity can itself be considered a stressor (Koch et al., 2017). Glucocorticoids impact on metabolic functioning, linked to diseases also associated with shift work, including obesity, type 2 diabetes mellitus, and cardiovascular disease (Faraut, Bayon, & Leger, 2013; Puttonen, Harma, & Hublin, 2010). Moreover, excess glucocorticoid release has profound negative impacts on brain functioning, cognitive performance, synaptic plasticity and even on protein translational processes (de Kloet, Joels, & Holsboer, 2005; Tahmasebi, Khoutorsky, Mathews, & Sonenberg, 2018).

However, rhythms in glucocorticoid secretion are also necessary to maintain physiological functioning. The peak in glucocorticoid release has been shown to be associated with the onset of the active phase promoted formation of dendritic spines

in the mouse motor cortex following motor learning on the rotarod task, and the subsequent trough in glucocorticoid release was necessary to maintain and stabilize these newly formed spines (Liston et al., 2013). Much remains to be examined when it comes to the importance of the HPA-axis as a whole and the role of its rhythmicity on the health outcomes of shift work. Animal studies may provide insight into these mechanisms.

Cap-dependent translation in synaptic plasticity and metabolism

Cap-dependent translation has been primarily studied in the context of synaptic plasticity and cancer cell biology (see Bramham et al., 2016 for review). However, protein synthesis occurs throughout the body and is a highly energy-demanding process. Thus, it does not seem unlikely that cap-dependent translation could have important roles in peripheral tissues involved in regulating metabolism. Cap-dependent translation has been shown to be mediated by metabolic processes such as elevated blood glucose (Dennis, Shenberger, Stanley, Kimball, & Jefferson, 2013). It has also been shown that within the liver, S6K1 and its regulator mTOR oscillate in a circadian fashion, with implications for regulation of lipid metabolism (Cornu et al., 2014). Later, Lipton and colleagues (2015) showed that cap-bound BMAL1 downstream of mTOR globally enhances liver protein synthesis, in a circadian fashion, peaking during the active phase. Given these findings, it seems reasonable to hypothesize that BMAL1 may play a role in liver cap-dependent translation and that this could be important for liver functioning, or metabolic functioning in general. This potential role has not yet been studied.

4.2.2 How do central and peripheral timing signals converge and communicate?

As shown in the previous section, shift work impacts several physiological functions with potential implications for health. However, there are numerous additional potential links between rhythm disturbance and health outcomes not directly studied in the present experiments. The circadian system itself is organized in a hierarchical structure, with the core clock of the SCN at the top, setting and synchronizing all other bodily rhythms. The SCN controls output rhythms by regulation of behavior,

physiological, and endocrine mechanisms. Mechanistically, this is achieved through the control of neuroendocrine and autonomic pathways. These mechanisms, as well as the integration of and communication between output rhythms, and their potential feedback on the SCN, have been extensively studied, but are not yet fully understood. Some of these will be discussed in the following section.

The SCN directly innervates numerous nuclei, primarily within the hypothalamus itself, and regulates output rhythms through both direct and indirect pathways (Kalsbeek et al., 2006). For example, the SCN controls glucocorticoid release through at least two separate mechanisms, one directly controlling the release of adrenocorticotropic hormone (ACTH), and another indirectly modulating adrenal sensitivity to ACTH (Buijs et al., 1999; Kalsbeek et al., 2006). As discussed in the previous section, glucocorticoid release and rhythmicity has importance for various physiological functions, including synaptic plasticity and cognitive functioning.

Another central rhythmic neuroendocrine output is melatonin. The SCN regulates the pineal release of melatonin through a multisynaptic pathway via the sympathetic branch of the autonomic nervous system (Kalsbeek et al., 2006). A potential role for rhythmic melatonin release in the regulation of synaptic plasticity has been suggested (Gerstner & Yin, 2010), as both visuospatial cognitive performance and LTP have been shown to be impaired after distribution of melatonin (Soto-Moyano et al., 2006). A recent study showed that spatial learning on an 8-arm radial arm maze task was enhanced in wild-type mice during the light phase, but this enhancement depended on nighttime elevation of melatonin (Jilg et al., 2019). Melatonin receptor knockout mice exhibited reduced spatial learning overall (Jilg et al., 2019). The melatonin receptor has also been implicated in individual variation in shift work tolerance, which will be discussed in section 4.3.1.

In paper I, body temperature was measured in the context of energy expenditure, but body temperature regulation is also of interest in the context of circadian rhythm regulation. In paper III, the observed reduction of the body temperature rhythm amplitude was clearly masked by the imposed forced activity protocol, and thus the

result primarily serves to show that resistance to either rhythm disturbance or masking varies between individuals. Nevertheless, body temperature is a factor of interest in the interaction between rhythmic systems. The SCN controls body temperature through autonomic pathways which are not yet fully elucidated (Morf & Schibler, 2013). The SCN itself is resistant to changes in body temperature, but body temperature serves as yet another way for the SCN to synchronize rhythms throughout the body (Mohawk et al., 2012). In the 1950s, scholars considered the possibility that body temperature fluctuations may directly impact cognitive performance (e.g. Kleitman & Jackson, 1950). While this does not appear to be the case (Carrier & Monk, 2000), recent work does suggest that fluctuations in brain temperature have implications for the regulation of synaptic plasticity (Frank, 2016; Wang, Wang, et al., 2014). While this primarily has been examined in a clinical context (i.e. with reference to potential neuroprotective effects of medically induced brain hypothermia), it is also possible that the daily rhythm of body temperature itself has implications for brain functioning.

There is still much to be discovered about the direct and indirect control of the SCN on rhythmic physiological properties. One example is how the SCN controls rhythmic hormones and tissues to maintain blood glucose homeostasis. These mechanisms are not yet well understood (see Kalsbeek, Yi, La Fleur, & Fliers, 2010 for review). Several other mechanisms may also be at play. In this work, neuroendocrine pathways involving e.g., thyroid-stimulating hormone have not been considered. Much recent work also implicates the gut microbiota in the regulation of clock functioning, a field which has further added to the intricacies of how different physiological systems interact and communicate (see Parkar, Kalsbeek, & Cheeseman, 2019 for review).

It should also be noted that while the SCN itself is relatively resistant to non-photic stimuli, the negative health consequences of shift work are likely to be results of a combination of rhythmic disturbances, involving both clock inputs and outputs. Several pathways between the SCN and rhythmicity in the brain and periphery, as well as the impact of such circadian disturbance on rhythms, and in turn on health,

remain to be elucidated. Only when these mechanisms and interactions are more clearly understood, may we begin to build and test working models which integrate the existing knowledge into a complete understanding of the health impacts of shift work and circadian disturbance in general.

4.2.3 Interaction between clock and homeostatic processes

In light of the present literature and the findings of paper III, the connection between clock and homeostatic processes cannot be ignored. The circadian and the homeostatic factors are frequently presented as separate processes in the context of sleep regulation, but in the real world, these are tightly intertwined, and virtually always interactive (Deboer, 2018). The most common health complaint of shift workers is poor sleep quality and reduced sleep length (Costa, 2015). The focus of this thesis has been on the importance of rhythms for adequate brain and metabolic functioning, but homeostatic factors are clearly also of great importance. Sleep may be required for adequate clock function, and vice versa.

Homeostatic and circadian processes interact to regulate the timing and quality of different sleep stages. REM sleep is strongly regulated by the circadian factor, and NREM sleep is primarily influenced by the homeostatic factor (Cambras et al., 2007; Dijk, 2009; Dijk & Czeisler, 1995). However, some work indicates that both incidence, amplitude, and frequency of slow waves are also impacted by rhythms, peaking during the biological day, primarily in posterior and central brain regions (Lazar, Lazar, & Dijk, 2015). Work from the present laboratory has also shown that relative to simulated day shift work, rats exposed to simulated night shift work exhibit enhanced slow-wave activity during wakefulness on simulated work shifts, despite modest effects on time spent in NREM sleep (Grønli et al., 2017), a finding that was replicated in paper III. The main effect of simulated night shift work on sleep was observed in terms of sleep consolidation, with shorter average NREM sleep bouts following simulated night shift work compared to simulated day shift work, although this was not replicated in paper III. Thus, the observed change in waking EEG, as evident by enhanced accumulation of slow-wave activity during quiet wakefulness during simulated night shift work, may at least in part be a result of

altered circadian modulation. Indeed, a mathematical modeling approach to understanding the effects of simulated night shift work on sleep in the present model suggested that the short NREM sleep bout duration observed by Grønli and colleagues (2017) primarily reflected a circadian effect (Rempe et al., 2018).

Several studies do suggest that in terms of sleep regulation, circadian, and homeostatic factors impact on one another. Activity within the SCN differs depending on sleep state (Deboer, Vansteensel, Detari, & Meijer, 2003). Moreover, sleep deprivation can modulate circadian activity. One study recently showed that phosphorylation of proteins within mouse forebrain synaptoneurosomes cycled in a circadian fashion, but that virtually all rhythmicity (98%) was lost after 4 hours of sleep deprivation (Bruning et al., 2019). Sleep deprivation has also been shown to reduce the circadian system's responsivity to light-induced phase shifts in the mouse (Challet, Turek, Laute, & Van Reeth, 2001). The relationship between circadian and homeostatic processes appears to be reciprocal, with high-amplitude circadian rhythmicity facilitating a period of deep sleep, which in turn contributes to maintaining rhythm strength (Deboer, 2018).

The results of paper III show that both aspects of daily rhythmicity and sleep drive serve to predict spatial memory performance and markers of brain functioning after simulated shift work. Homeostatic factors could also contribute to the *metabolic* effects associated with shift work in both human and animal models. Both short and long sleep duration have been associated with metabolic diseases, including obesity and type 2 diabetes (Taheri, 2006; Tan, Chapman, Cedernaes, & Benedict, 2018). Several important metabolic functions occur during sleep, including the release of growth hormone associated with slow-wave sleep (Davidson, Moldofsky, & Lue, 1991). Sleep restriction also reduces both overall circulating levels, and diurnal rhythm amplitude, of the satiety hormone leptin, despite no change in caloric intake (Spiegel et al., 2004). Recent work has also shown changes in brain metabolic rate in different stages of sleep and wakefulness in the mouse (Rempe & Wisor, 2014). Timing of food intake may impact on homeostatic processes. One study showed that feeding during the rest phase induced internal desynchrony between hypothalamic

regions involved in the regulation of energy balance, sleep, and arousal (Ramirez-Plascencia et al., 2017). Another study suggested that the dietary content of food may even impact on homeostatic processes; a chronic high-caloric diet in mice resulted in a slower wake-induced increase in homeostatic sleep pressure (Panagiotou, Meijer, & Deboer, 2018). Given these lines of research, it appears relevant to hypothesize that homeostatic and circadian factors interact to promote metabolic dysregulation associated with shift work. Future studies should design experimental protocols to assess the separate contribution and potential additive effects of the two processes on metabolic outcomes.

4.3 Relevance, impact, and future directions

4.3.1 Shift work tolerance

The notion that some individuals handle shift work poorer than others has been discussed, and used as the basis for legislation, since the dawn of industrialization. Several traits, such as young age, male gender, and late chronotype have been linked to a higher propensity for shift work tolerance (Saksvik et al., 2011). Likewise, personality traits such as high flexibility and extraversion, and low neuroticism have been linked to higher shift work tolerance (Saksvik et al., 2011). Shift work tolerance has not previously been considered in animal models, but the results of paper III indeed suggest individual variations in spatial memory performance and markers of brain function after simulated shift work. This variation was in part predicted by aspects of daily rhythmicity and sleep drive. Of relevance, the tendency for circadian desynchronization has been associated with lower shift work tolerance in humans (Reinberg & Ashkenazi, 2008). This finding serves at least some parallel to the results of paper III. The link between shift work tolerance and sleep is somewhat more unclear. Workers who self-reported lower shift work tolerance also reported increased sleepiness and less sufficient sleep but did not exhibit shorter or poorer sleep when measured objectively (Axelsson et al., 2004). Likewise, in paper III, parameters related to time spent in sleep did not predict markers of brain functioning

or spatial memory performance. Only markers of sleep drive, including waking function, predicted these outcomes.

Genetics

Human studies are also identifying genetic markers of shift work tolerance. Sookoian and colleagues (2007) showed an association between shift work and the serotonin transporter promoter gene *SLC6A4*. The short variant of *SLC6A4*, associated with reduced platelet serotonin, was more frequent in rotating shift workers, especially among those who had longer than 60 months of rotating shift work, compared to day workers in the same factory. Another study found an increased risk of insomnia among shift workers carrying the short variant of the *SLC6A4* gene mutation (Pallesen, Jacobsen, Nielsen, & Gjerstad, 2019). Serotonin has been proposed as an intermediate between metabolic dysfunction and circadian disturbance (Versteeg, Serlie, Kalsbeek, & la Fleur, 2015). Another study showed an association between variants of both the *SLC6A4* and the *catechol-O-methyltransferase* (*COMT*) genes on chronotype (Ojeda et al., 2014), which in turn is associated with shift work tolerance (Åkerstedt, 1998). Interestingly, the *COMT* gene is associated with the metabolism of dopamine in the brain and has been shown to be important for synaptic plasticity, learning, and memory (Egan et al., 2001). However, a later study failed to replicate the association between the *COMT* gene variant and chronotype (Jawinski et al., 2016). The role of these genetic variants is not clear, and their role specifically regarding shift work tolerance remains to be investigated in larger-scale studies.

The functioning of the circadian rhythm hormone melatonin has also been related to shift work tolerance. In a genome-wide association study, Sulkava and colleagues (2017) identified a genetic variant near the *melatonin receptor 1A* gene, possibly related to reduced brain melatonin signalling, associated with symptoms of job-related exhaustion in shift workers. Taken together, genetic studies serve as examples of how basic biological functions may relate to shift work tolerance. Although the findings of the present experiments in rats cannot be directly translated to human shift workers, the idea that one in the future might be able to use e.g. genetic testing to predict how individuals may tolerate shift work is an attractive one. Additionally,

animal work can aid in elucidating the mechanisms by which these genetic mutations impact on shift work tolerance and related factors.

4.3.2 Future directions: Mitigating the negative effects of night shift work

Hopefully, an enhanced understanding of the pathways and mechanisms that lead to negative health effects of shift work, may contribute to inform future efforts to mitigate these negative effects. This section examines some of the most prominent countermeasures at present, in light of the present work and the related literature.

Countermeasures to limit circadian misalignment on the night shift

Countermeasures to combat the negative effects of night shift work often aim to address the circadian misalignment that has been associated with night shift work, also in the present work. Approaches typically attempt to either use measures to reduce circadian misalignment or to enhance circadian rhythm adaptation to the work shift. One approach is to limit shift work schedules to include no more than 2-3 night shifts in a row (Burgess, 2007), or to restrict food intake on the night shift (Lowden et al., 2010). However, considering the potential impact of food and nutrition on brain functioning and cognitive performance, as discussed in section 4.1.2, one might exercise caution before recommending overnight fasts on the night shift to individuals in physically and/or mentally demanding professions.

An individualized approach to promote congruence between the circadian rhythm and the shift schedule might take individual variation into account. So far, chronotype has been the primary individual variable considered. The literature suggests that individual differences in chronotype impact on the tendency to desynchronize rhythms in response to different types of shift schedules. For example, late chronotypes tend to suffer more sleep difficulties following the morning shift, while it is the early chronotypes who tolerate the night shift most poorly (Åkerstedt, 1998). One study showed that advancing the rhythm of late chronotypes through timed light exposure, meals, caffeine and exercise improved mental health and performance at the beginning of a day shift (Facer-Childs, Middleton, Skene, & Bagshaw, 2019). However, in some sectors, a more attractive countermeasure both for workers and

employers could be to adapt work schedules to the individual's chronotype. One study showed that when chronotype was matched to the work schedule, self-reported sleep quality was improved and measures of circadian misalignment were reduced (Vetter, Fischer, Matera, & Roenneberg, 2015).

Countermeasures to enhance waking functioning on the night shift

Light is used to induce phase-shifting but may also have independent alerting effects to promote cognitive functioning (Grønli & Mrdalj, 2018). Blue-light sensitive ipRGC photoreceptors of the retina not only signal directly to the SCN but also indirectly to cortical centers important for learning and cognition, such as the hippocampus and the PFC (Fernandez et al., 2018). One recent study in humans showed that bright light, compared to standard light, during simulated night shift work enhanced cognitive performance and circadian adaptation to the night shift schedule (Sunde, Mrdalj, et al., 2020). Moreover, blue-enriched light compared to standard white light enhanced cognitive performance, but without additional effects on circadian adaptation (Sunde, Pedersen, et al., 2020).

While light interventions are indeed beneficial to promote alertness, the long-term effects on e.g. metabolic health are less well known. Light at night is associated with metabolic dysfunction and weight gain, both in the short (Borniger, Maurya, Periasamy, & Nelson, 2014) and the long term (Plano et al., 2017). Light signals directly to the lateral hypothalamus, involved in regulating appetite and eating behavior (see Fonken & Nelson, 2014 for review). Studies in rodents may further elucidate the mechanisms by which light impacts on whole-body physiology. While white light is associated with the rest phase for nocturnal rodents, it appears that blue-enriched light might indeed be alerting to mice (Pilorz et al., 2016). The present rat model of shift work, with associated degraded waking function, reduced cognitive performance on the MWM, and impaired protein markers of brain function might serve as an excellent model on which to test the proposed alerting effects of blue-enriched light on nocturnal rodents.

Several other countermeasures to promote cognitive functioning on the night shift have been proposed, either by dissipating or overriding the gradually increasing homeostatic sleep drive throughout the night shift. Napping itself reduces homeostatic sleep pressure, and hence reduces sleepiness, but may induce sleep inertia, resulting in temporary cognitive impairment (Ruggiero & Redeker, 2014; Zion & Shochat, 2019). Considering the interrelation between clock and homeostatic processes, manipulating sleep pressure may not only impact on sleep drive. Dissipated sleep pressure has been shown to increase sensitivity to the phase-shifting effects of light (Challet et al., 2001) and it may also enhance sensitivity to the alerting effects of light, although this remains to be tested.

Lastly, pharmacological interventions might be utilized to enhance cognitive performance by modulating the clock, homeostatic sleep drive, or both. Some interventions include the administration of wake-promoting drugs such as Modafinil to enhance waking functioning on the night shift (Hart et al., 2006; Wisor, 2019), but the use remains controversial. The most used stimulant to counteract cognitive disturbance associated with shift work is caffeine. Caffeine enhances waking performance but can reduce sleep quality and length (O'Callaghan, Muurlink, & Reid, 2018). Interestingly, caffeine has additionally been shown to increase sensitivity to the phase-shifting effects of light (van Diepen et al., 2014). Specific recommendations typically advise limiting caffeine consumption to the first part of the night shift. Still, little is known about the effects of these countermeasures on the circadian and homeostatic systems, and physiology in general. There is a need for more animal work to further investigate these effects.

4.4 Limitations and model validity

4.4.1 Methodological limitations

Specific limitations of the experiments and results reported in this thesis have been discussed in papers I, II, and III. In this section, some more general and overarching limitations relating to sample size, statistical power, and causal inference are discussed.

When conducting animal experiments, the choice of the number of animals must be taken while considering both statistical and ethical aspects. The three R's urges the reduction of the number of animals used to achieve a certain amount of information (Russell & Burch, 1959). Therefore, animal studies commonly have small sample sizes, which may challenge traditional statistical hypothesis testing and the assumptions underlying such tests. Still, the number of animals must be sufficient to yield the desired statistical power to allow generalizations. The American Statistical Association has urged the reporting of measures of magnitude rather than merely of statistical significance (Wasserstein & Lazar, 2016; Yaddanapudi, 2016) and has even recommended the abandonment of the practice of dichotomizing results into statistically significant or non-significant based on p-value cut-offs (Wasserstein, Schirm, & Lazar, 2019).

The p-value is a continuous measure of compatibility between the observed data and the test (null) hypothesis under a set of assumptions (Rafi & Greenland, 2020). In other words, the p-value is the probability of obtaining data similar to, or more extreme than, the observed, assuming that the model, including the null hypothesis, is true in the population. Misinterpretations of p-values and statistical significance are widespread in the literature (Greenland et al., 2016). Therefore, the results of the present experiments are primarily reported in terms of effect size and explanatory power (fold change, Cohen's d, R^2, and adjusted R^2). The fact that most of the measures in the present studies yielded large effect sizes and explanatory power, despite small sample sizes and lack of statistical significance, indicates that the methods used were sufficiently sensitive to identify consequences of the present model of shift work.

Another consequence of the strive toward reducing the number of animals used in scientific studies is the use of strategies to minimize individual differences and data variability, e.g., by using inbred strains of animals. In the present project, a different approach taking advantage of individual variability within the data in the outbred rat strains was chosen. The data variability was used to identify predictors of model outcomes using hierarchical linear regression. The choice of hierarchical regression

modeling may be considered somewhat unorthodox, as this method is most commonly used in clinical and epidemiological studies, and not in animal studies. Moreover, as the choice and ordering of predictors were made based on domain knowledge, the analysis contains some inherent bias. Therefore, the outcomes of the hierarchical regression analysis should be primarily considered for purposes of generating hypotheses to be experimentally tested in future studies, and not for causal inference.

4.4.2 Evaluating model validity

To quote British statistician George Box: "All models are wrong, but some models are useful" (Box & Luceño, 1997). Animal models are not intended to replicate the human experience, but rather to extract certain factors assumed to be of importance and allow inferences that would be impractical or impossible to draw from studies in humans (Ericsson, Crim, & Franklin, 2013). In other words, animal models are not intended to perfectly replicate a complex situation but rather act as simplified representations of reality. Still, knowing that an estimated 90% of behavioral neuroscience findings do not replicate to humans (Garner, 2014), it is important to properly evaluate animal models with respect to validity, to identify future lines of study where more data is needed, and to identify aspects where a model lacks validity and should be disregarded. Belzung and Lemoine (2011) suggest five criteria for evaluating the validity of animal models, namely homolog, pathogenic, mechanistic, face, and predictive validity. In this section, the present model of shift work will be evaluated along these criteria.

Homological validity refers to whether the choice of species and/or strain is appropriate for the modeling of the given phenomenon. In the present model, the rat has been chosen to model and measure human characteristics such as metabolism, cognitive performance, biochemical markers of brain function, body temperature rhythm, sleep, and waking function. There are known differences in metabolic rate and metabolic responses to stress between rats and humans (Cohen, Janicki-Deverts, & Miller, 2007; Demetrius, 2005), which should be considered when evaluating the outcome of the present experiments. Still, the circadian system's control of metabolic

processes and hence the effects of circadian misalignment on metabolic functioning appears to be relatively similar in humans and rats (Johnston, Ordovas, Scheer, & Turek, 2016). When considering cognitive and brain functions, the human cortex, and especially the human prefrontal cortex is much larger relative to the rest of the brain, compared to rats. As far as we know, humans are capable of far more complex cognitive feats and likely rely more on prefrontal functions than rats do. Still, the rat has been the preferred model animal for tests of cognitive ability and synaptic plasticity due to its ability and motivation to learn and perform complex tasks, and since the size of its brain (large relative to e.g. the mouse) makes it possible to study the functions of several brain structures both *in* and *ex vivo*.

Another issue when assessing the homological validity of the present model relates to the use of a nocturnal species to model a phenomenon based on circadian misalignment in a diurnal species. It becomes important then to distinguish where in the circadian hierarchy diurnality or nocturnality appears. The rhythmicity within the human and rat SCN are very similar in terms of phase when examining a range of measures including rhythms of metabolic rate, neurotransmitter release, and clock gene expression (Smale, Lee, & Nunez, 2003). Thus, while specific nocturnality or diurnality appears downstream of the SCN, the regulation of circadian rhythmicity between species is similar at the core (see Kalsbeek et al., 2006 for further discussion of the mechanisms downstream of the SCN which give rise to diurnality and nocturnality).

Pathogenic validity and mechanistic validity assess the processes which lead to dysfunction, in this case of metabolic and brain physiology. Specifically, pathogenic validity refers to the processes which trigger dysfunction, while mechanistic validity refers to more general mechanisms contributing to disturbance. Since we do not know the precise factors which *trigger* negative health outcomes in shift workers (these are likely highly complex), we can only evaluate the present model with reference to mechanistic validity. Both human and animal data can be appraised and evaluated mechanistically within the broad context of circadian misalignment, as has been the major theoretical framework of this thesis. Both human and animal studies seem to

suggest that shifted timing of food intake contributes to metabolic dysfunction observed in human shift workers (see section 4.1.1). However, further studies are required to test whether the identified potential neural mechanisms (e.g. reduced translational activity within the prefrontal cortex) also have relevance for humans.

Face validity refers to whether the observed effects in an animal model are the same as those observed in humans. Most observed outcomes of the present animal model are in line with observations in humans, indicative of face validity. In the reported experiments we observe several outcomes associated with human shift work, including a shift in the timing of food intake which stabilizes after three days of simulated shift work, dysregulation of lipid metabolism, circadian misalignment, reduced circadian rhythm amplitude, reduced cognitive functioning, and reduced waking function, without effects on total sleep time.

Lastly, predictive validity refers to the ability of a model to predict aspects of a phenomenon in humans that are currently not known. The outcomes of the experiments presented in this thesis allow several predictions to be made in future experimental animal studies and human studies, but the testing of face validity remains to be done. Although it might be difficult to measure e.g. cap-dependent translational activity within the prefrontal cortex of humans on the night shift, brain imaging methods such as magnetic resonance (MR) spectroscopy are being improved and might allow the measurement of relevant biochemical substrates within different brain structures across time-points in humans. In addition, the findings of paper III allow the prediction that individual variation in markers of sleep drive should impact more on cognitive performance after simulated shift work than the degree of disturbance to daily rhythms. This prediction could be relatively easily investigated in humans.

4.4.3 Reductionism

One concept remaining to discuss related to the evaluation of animal models concerns reductionism. All animal models are inherently reductionist. When reducing a concept to smaller parts there is always a risk that one might be missing out on some

unifying complexity of a situation which in itself has effects on the observed outcomes (see Cohen et al., 2007 for discussion). However, while some models are designed to test precise basic phenomena, such as the use of LTP to model synaptic plasticity, the present model uses a relatively non-specific intervention, namely forced activity to model human shift work. This means that the experiments reported on in this thesis are not appropriate to make strong inferences about the specific mechanistic pathways which cause negative health effects in shift workers. The present model provides somewhat of a middle-ground. Shift work is an extremely complex phenomenon that induces multifaceted effects in virtually every physiological system studied. Hopefully, the results of the present model can provide novel hypotheses that can be studied at many levels of investigation, ranging from the societal, cultural, and social levels, down to the levels of cells, proteins, and genes, both in humans and animals.

4.5 Concluding remarks

The work presented in this thesis has demonstrated that just 3-4 consecutive days of simulated night shift work have detrimental effects on metabolic and brain functioning. Moreover, individual variation in spatial memory performance and markers of synaptic plasticity were predicted by different aspects of work, daily rhythmicity, and sleep drive. There is much that is unknown about how the circadian system drives daily rhythmicity in different tissues, how daily rhythms interact, and how circadian misalignment contributes to the negative health effects associated with shift work. Still, the results presented herein show that the contributing factors are complex, particularly implicating circadian rhythmicity in specific aspects of brain functioning. These findings may allow the further elucidation of how circadian misalignment impacts all aspects of health, both in those who are engaged in shift work, as well as in other populations.

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Shift in Food Intake and Changes in Metabolic Regulation and Gene Expression during Simulated Night-Shift Work: A Rat Model

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Abstract: Night-shift work is linked to a shift in food intake toward the normal sleeping period, and to metabolic disturbance. We applied a rat model of night-shift work to assess the immediate effects of such a shift in food intake on metabolism. Male Wistar rats were subjected to 8 h of forced activity during their rest (ZT2-10) or active (ZT14-22) phase. Food intake, body weight, and body temperature were monitored across four work days and eight recovery days. Food intake gradually shifted toward rest-work hours, stabilizing on work day three. A subgroup of animals was euthanized after the third work session for analysis of metabolic gene expression in the liver by real-time polymerase chain reaction (PCR). Results show that work in the rest phase shifted food intake to rest-work hours. Moreover, liver genes related to energy storage and insulin metabolism were upregulated, and genes related to energy breakdown were downregulated compared to non-working time-matched controls. Both working groups lost weight during the protocol and regained weight during recovery, but animals that worked in the rest phase did not fully recover, even after eight days of recovery. In conclusion, three to four days of work in the rest phase is sufficient to induce disruption of several metabolic parameters, which requires more than eight days for full recovery.

Keywords: shift work; night work; animal model; metabolism; circadian rhythmicity; gene expression; body temperature; body weight; food intake

1. Introduction

Working shifts is common in modern societies [1], despite the fact that chronic night-shift work is linked to an increased risk for a wide variety of diseases, including metabolic disorders such as obesity and diabetes [2–8].

The regulation of daily rhythmicity in physiology and behaviour is closely intertwined with the regulation of metabolism. The circadian clock in the suprachiasmatic nuclei (SCN) of the

hypothalamus influence other hypothalamic regions and peripheral tissues involved in regulation of feeding behaviour, metabolism, energy storage, and energy breakdown [9].

An important function of the endogenous circadian system is that it allows for anticipation of meals, and thereby facilitates efficiency in metabolic regulation [10]. The activity of organs involved in food processing and energy metabolism—including the digestive tract [11], liver [12,13], and pancreas [14]—shows clear circadian rhythmicity. Food intake is thought to be one of the primary zeitgebers (time-givers) for the coordination and timing of these rhythms [15].

Night-shift workers are exposed to conflicting zeitgebers, imposed by changes in food intake and activity patterns, as well as nocturnal light exposure [16–18]. Consequently, during night-shift work, endogenous circadian rhythms become desynchronized, as conflicting signals are sent to different tissues and organs. Often, the desynchronization is prolonged, as entrainment of rhythms in different tissues occurs at different rates [10].

The effects of working at night are immediate, and although humans may partially adapt to the night-shift, many physiological systems fail to adjust [19]. In Norway and Europe generally, night workers commonly work three to four night shifts in a row. Data from a simulated night-shift study in humans indicate that the metabolic effects are most pronounced during the initial two days, followed by compensatory mechanisms triggered by negative metabolic effects [20]. Ribeiro and colleagues induced a 9 h phase delay in a human simulated shift work study. Controlling energy and macronutrient intake, they showed that plasma levels of free fatty acids and triglycerides were reduced postprandially, indicative of poor metabolic regulation, but that this reduction was partially normalized on the third day following the phase delay [20].

Animal models of shift work may provide important clues to the mechanisms by which shift work causes metabolic disruption. Although several animal models indicate marked metabolic changes due to rest-work [21], only models involving relatively extreme rest-work schedules have been applied so far. The early metabolic effects of simulated night-shift work have yet to be investigated in animal models.

In the present study, we utilized a rat model of shift work to investigate the early effects of simulated night work on metabolism. We hypothesized that a shift in feeding rhythm during work hours occurs during a four-day rest-work schedule, as a sign of metabolic compensation. Specifically, we proposed that compensatory mechanisms triggered by negative metabolic effects would be displayed in liver gene expression due to a shift in the timing of food intake.

2. Materials and Methods

2.1. Ethical Approval

This project was approved by the Norwegian Animal Research Authority (permit number: 2012463) and performed according to Norwegian laws and regulations, and The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

2.2. Animals and Housing

Adult male rats (n = 40, Wistar, NTac:WH, Taconic, Silkeborg, Denmark) weighing approximately 300 g at arrival, were group housed in individually ventilated cages (IVC, Tecniplast, Buggugitate, Italy, 75 air changes/h) type IV (480 × 375 × 210 mm, 1500 cm^2). After surgery and during the experiment, animals were single housed (IVC cage type III, 425 × 266 × 185 mm, 800 cm^2). The study was performed under a 12 h light/12 h dark (LD) cycle (lights on at 06:00, zeitgeber time 0; ZT0). Lights were gradually dimmed on and off over a period of 1 h, and were fully on at 07:00 and fully off at 19:00. Food (rat and mouse No. 1, Special Diets Services, Witham, Essex, UK) and water were provided ad libitum throughout the experiment.

2.3. Study Design

To assess changes in food timing, rats were randomly assigned to one of two treatment groups, either rest-work (RW, $n = 24$) or active-work (AW, $n = 16$). The experiment first consisted of four days of undisturbed baseline, followed by a four-day work period. A subset of animals was monitored during eight days of undisturbed recovery (RW $n = 14$, AW $n = 11$). See Figure 1 for a graphical overview of the study design. A shift in feeding rhythm of RW animals was observed during work session three. For assessment of changes in metabolic gene expression in the liver during this shift, another experiment was conducted in a subset of animals (RW $n = 10$, AW $n = 10$) after five weeks of recovery from the initial four-day shift-work period. Since the animals were still young adults [22], we consider it unlikely that ageing affected the results of this second experiment. The animals were randomly assigned to three consecutive days of either AW or RW as described above, euthanized after the third work session, and compared to time-matched non-working controls ($n = 5$, each condition).

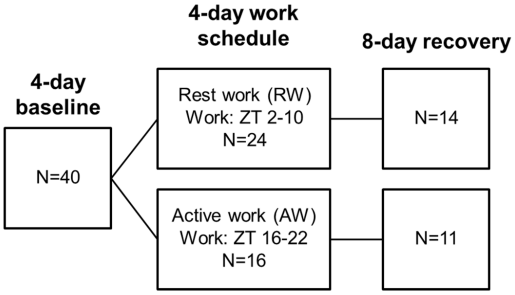

Figure 1. Overview of the study design. Animals were monitored for four baseline days, and a four-day work schedule during which animals were exposed to either rest-work or active work. The work schedule was followed by eight days of recovery. After at least five weeks of recovery, a subset of animals underwent a three-day work schedule (not shown) before euthanasia and tissue harvest from experimental animals and undisturbed time-matched controls.

2.4. Simulated Shift Work Procedure

To mimic human shift work and compare this with normal daytime work, rats were exposed to forced activity for 8 h per day, centred either during the rats' normal active phase (AW; ZT 14-22) or during the rats' normal rest phase (RW; ZT 2-10), as described previously [23]. Forced activity was achieved by placing the rats in automatically rotating wheels (Rat Running Wheel, TSE running wheel system, Bad Homburg, Germany; 24 cm diameter; 3 rpm; 1440 revolutions or 1.086 km of linear distance per 8 h session). Food and water was available ad libitum. Rotating wheels, feeders, and water bottles were cleaned after each work session with a 5% ethanol solution. Between sessions, animals were housed in their home cages.

2.5. Telemetric Recording and Analyses of Body Temperature

Rats were implanted with transmitters (Physiotel, Data Sciences International, St. Paul, MN, USA) for continuous wireless recording of body temperature, as previously described [24]. In brief, animals were anaesthetized with subcutaneous injection of a mixture of fentanyl 0.277 mg/kg, fluanizone 8.8 mg/kg, and midazolam 2.5 mg/kg (Hypnorm, Janssen, Beerse, Belgium; Dormicum, Roche, Basel, Switzerland; Midazolam Actavis, Actavis, Parsippany-Troy Hills, NJ, USA), and the transmitters were placed in subcutaneous pockets in the dorsomedial lumbar region (4ET transmitters) or in the neck region (F40-EET transmitters). Animals were allowed to recover for 14 days before entering the experiment. Body temperature was recorded every 10 s, at 50 Hz sampling rate, and signals were collected with Dataquest ART software (version 4.1, Data Sciences International, St. Paul, MN, USA).

Chronos-Fit software (Heidelberg University, Heidelberg, Germany) [25] was used for linear analyses of body temperature. From the linear analysis, 24 h mean, 12 h rest phase mean (lights on; ZT 12–24), and 12 h active phase mean (lights off; ZT 0–12) were calculated.

2.6. Body Weight and Food Intake Measurements

At baseline, all animals were weighed to assess 24 h and four-day body weight change. Baseline food and water intake were monitored across an 8 h window equal to the length of one work session, and across a 16 h window equal to the time between each work session. During the four-day work period, body weight change, food intake, and water intake were monitored for each 8 h work session and the 16 h between the work sessions. During the eight-day recovery phase, body weight change was monitored every four days.

2.7. Assessment of Metabolic Gene Expression in the Liver

Following the third work session, animals were fasted for 2 h to avoid the immediate effects of food intake on gene expression, anaesthetized with isoflurane, and sacrificed by decapitation. AW were sacrificed at ZT0, before the transition from dark to light phase, and RW at ZT12, before the transition from light to dark phase. A separate group of undisturbed animals never exposed to simulated work were used as time-matched controls, and sacrificed at the same zeitgeber times as experimental animals (AW control: ZT0; and RW control: ZT12). Liver tissue was harvested, flash frozen, and stored at −80 °C until analysis. Samples were homogenized using a TissueLyser (Qiagen, Valencia, CA, USA). RNA extraction was performed using a 6100 Nucleic acid PrepStation (Applied Biosystems, Foster City, CA, USA). A total of 20 ng RNA was transcribed to cDNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). Real-time polymerase chain reaction (PCR) was run on the Applied Biosystems 7900 Real-Time PCR System, with each sample run in triplicate. Relative gene expression levels were determined using the comparative ΔCt method, using β-actin (Actb) and ribosomal protein lateral stalk subunit P0 (Rplp0) as endogenous controls. Sequence names, main function, accession numbers, and primer sequences are shown in Table 1.

2.8. Statistical Analyses

Statistical analyses were conducted using STATA (release 14; StataCorp LP, College Station, TX, USA). Baseline food intake and body weight were compared between groups using student's *t*-test (two-tailed). For all other statistical analyses of food intake, body weight, and body temperature, we used mixed model analysis using restricted maximum likelihood estimation with the unstructured covariance between random effects. Where significant effects were observed, pairwise comparisons of groups at each time point were performed as well as comparing each day to baseline. Difference in gene expression (fold change) between groups was evaluated using student's *t*-test (two-tailed). Statistical significance was accepted at $p < 0.05$.

Table 1. Sequence names, accession numbers, and primer sequences for genes examined for hepatic transcriptional levels.

Gene	Main Function	Accession Number	Forward Primer	Reverse Primer
Fatty acid synthase (*Fasn*)	Fatty acid synthesis	NM_017332	CCATCATCCCCTTGATGAAGA	GTTGATGTCGATGCCTGTGAG
Stearoyl-CoA 9-desaturase; Stearoyl-CoA desaturase 1 (*Scd1*)	Fatty acid desaturation	NM_139192	TCAATCTCGGGAGAACATCC	CATGCAGTCGATGAAGAACG
Diacylglycerol O-acyltransferase 1 (*Dgat1*)	Triglyceride synthesis	NM_053437	AATGCTGCGGAAAAACTACG	TTGCTGGTAACAGTGCTTGC
Diacylglycerol O-acyltransferase 2 (*Dgat2*)	Triglyceride synthesis	NM_001012345	AATCTGTGGTGCCGCCAG	TCCCTGCAGACACAGCTTTG
Glycerol-3-phosphate acyltransferase 1, mitochondrial (*Gpam*)	Triglyceride synthesis	NM_017274	AATGCTGCGGAAAAACTACG	TTGCTGGTAACAGTGCTTGC
Sterol regulatory element-binding protein 1c (*Srebf1*)	Key regulator of fatty acid/triglyceride synthesis	NM_001271207	GAACCGCAAAGGCTTTGTAAA	ACCCAGATCAGCTCCATGGC
Hydroxymethylglutaryl-CoA synthase, cytoplasmic (*Hmgcs1*)	Sterol synthesis	NM_017268	CAGCTCTTGGGATGGACGA	GGCGTTTCCTGAGGCATATATAG
Sterol regulatory element-binding protein 2 (*Srebf2*)	Key regulator of sterol synthesis	NM_001033694.1	GCCGCAACCAGCTTTCAA	CCTGCTGCACCTGTGGTGTA
Peroxisome proliferator-activated receptor alpha (*Ppara*)	Fatty acid oxidation	NM_013196	AATGCAATCCGTTTTGGAAGA	ACAGGTAAGGATTTCTGCCTTCAG
Peroxisome proliferator-activated receptor gamma (*Pparg*)	Adipocyte differentiation	NM_001145366	CCACAAAAAGAGTAGAAATAAATGTCAGTAC	CAAACCTGATGGCATTGTGAGA
Insulin receptor substrate 2 (*Irs2*)	Mediation of insulin effects	NM_001168633	GAAGCGGCTAAGTCTCATGG	CTGGCTGACTTGAAGGAAGG
Phosphorylase, glycogen, liver (*Pygl*)	Glycogen breakdown	NM_022268	AAAAGCCTGGAACACAATGG	TCGGTCACTGGAGAACTTCC
Mechanistic target of rapamycin (*Mtor*)	Mediation of cellular metabolic stress response	NM_019906	CATGAGATGTGGCATGAAGG	AAACATGCCTTTGACGTTCC
Carbohydrate responsive element binding protein (ChREBP/Mlxipl)	Triglyceride synthesis in response to carbohydrates	AB074517	CTCTCAGGGAATACACGTCTCC	ATCTTGGTCTTTGGGTCTTCAGG
β-actin (*Actb*)	Endogenous control	NM_031144	TACAGCTTCACCACCACAGC	CTTCTCCAGGGAGGAAGAGG
Rattus norvegicus ribosomal protein lateral stalk subunit P0 (*Rplp0*)	Endogenous control	NM_022402.2	CATTGAAATCCTGAGCGATGT	AGATGTTCAACATGTTCAGCAG

3. Results

3.1. Baseline Parameters

At baseline, the two groups on average had similar body weight and food intake ($p > 0.09$ in all cases). The absolute baseline food intake across 24 h was 23.1 (±0.53 g) for RW and 21.36 (±0.70 g) for AW. Eight hours baseline food intake was 4.39 (±0.37 g) for RW and 8.54 (±0.70 g) for AW. Moreover, there were no significant differences in body temperature parameters (24 h mean, active phase mean, rest phase mean; $p > 0.10$ in all cases). Baseline day three was used as baseline reference in the subsequent analyses.

3.2. Feeding and Body Temperature Rhythms, during and after One Rest-Work Period

In RW, total 24 h food intake was significantly reduced on all four work days compared to baseline ($p < 0.001$ for all days; Figure 2a), but not compared to AW. RW food intake gradually increased across the first two 8 h work sessions. On day three, food intake stabilized, and was significantly increased in the 8 h work sessions on both days three and four ($p < 0.02$; Figure 2b). Hence, RW shifted the timing of food intake on work day three. This shift was also significant compared to AW food intake during the work session on days three and four ($p < 0.01$, both work sessions; Figure 1b). Between work sessions, food intake was decreased on all work days ($p < 0.001$, all days) compared to baseline.

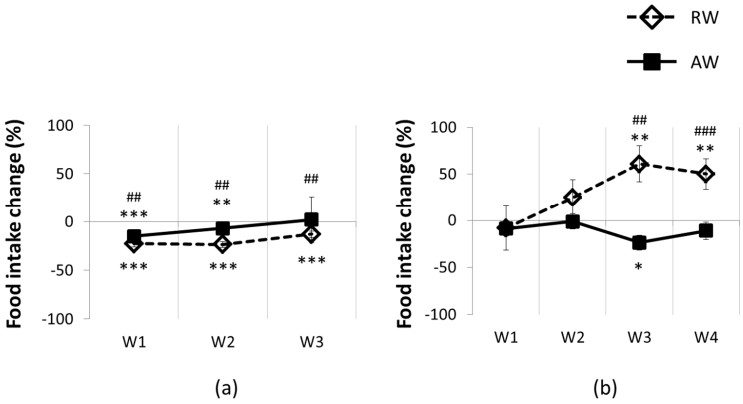

Figure 2. Food intake across one shift work period for active workers (AW) and rest-workers (RW): (**a**) 24 h food intake; (**b**) 8 h food intake (during work session). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W1–4 indicates work days 1 to 4. W4 not included in 24 h food intake due to missing measurements. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, compared to baseline. ## $p < 0.01$; ### $p < 0.001$, between groups.

RW body weight dropped and remained below baseline across the whole four-day rest-work period (Figure 3). The reduced body weight was evident across 24 h, and during the 8 h work sessions. RW gained less weight between the work sessions, both compared to baseline ($p < 0.001$, all work days) and to AW ($p < 0.008$, all work days). The most pronounced body weight loss was observed during work day two, before body weight loss appeared to attenuate (see Figure 3).

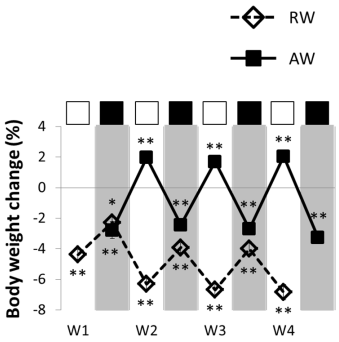

Figure 3. Body weight change during and between work sessions for active workers (AW) and rest-workers (RW). White rectangles indicate work hours for RW. Black rectangles indicate work hours for AW. Data are shown as percentage change relative to baseline. Shaded bars indicate lights off (active phase). Error bars indicate SEM. W1–4 indicates work days 1 to 4. * $p < 0.05$; *** $p < 0.001$, compared to baseline.

In RW, mean 24 h body temperature during the four-day rest-work period did not significantly differ from baseline or AW. However, RW body temperature was elevated during the 12 h rest phase (which now included the 8 h work session; $p < 0.001$ for all four work days), and was reduced during the 12 h active phase on work days two to four ($p < 0.003$ all three work days, Figure 4b). The 12 h body temperature increase from baseline during rest phase was more pronounced in RW than in AW on work days three and four ($p < 0.02$ both), and lower during the 12 h active phase on work day four ($p = 0.02$).

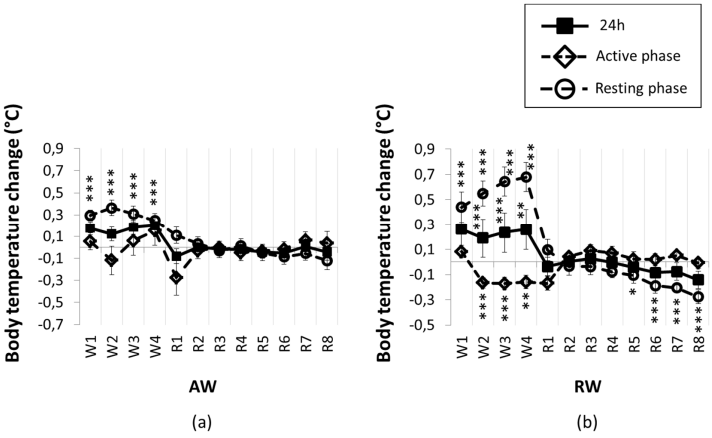

Figure 4. Mean body temperature during one shift work period (W1–4) and recovery (R1–8) for (**a**) active workers (AW) and (**b**) rest-workers (RW). Data are shown as mean percentage change relative to baseline. Error bars indicate SEM. W1–4 indicate work days 1 to 4. ** $p < 0.01$; *** $p < 0.001$, compared to baseline.

In AW, total 24 h food intake was reduced on work days one and two compared to baseline ($p < 0.05$, both days), but not on work days three to four (Figure 2a). During the 8 h work sessions, food intake was only significantly reduced on work day three compared to baseline ($p < 0.001$, Figure 2b). Food intake in the 16 h between work sessions was not significantly different from baseline.

AW body weight was reduced across 24 h ($p < 0.001$, all workdays) and during the 8 h work sessions ($p < 0.001$, all work sessions), but increased more during the 16 h between the work sessions ($p < 0.001$, all workdays) compared to baseline condition (Figure 3).

Mean 24 h body temperature during the four-day work period in AW was slightly elevated (0.17 °C on average), but not significantly different from baseline (Figure 4a). AW mean 12 h body temperature during the active phase (including the 8 h work session) was unaltered compared to baseline. Mean 12 h body temperature during the rest phase between work sessions was increased by 0.31 °C on average compared to baseline ($p < 0.001$, all days).

3.3. The Recovery Period

Rest-workers increased but did not regain the lost body weight during the 8 days recovery phase ($p < 0.001$; Figure 5). Mean body temperature for 24 h and for 12 h active phase was not significantly different from baseline, while mean body temperature during the 12 h rest phase progressively declined in the course of the recovery period, until it was significantly lower than baseline on recovery days five to eight ($p < 0.02$; Figure 4b).

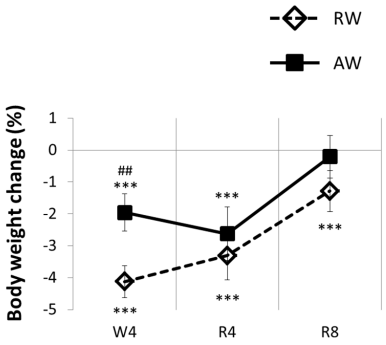

Figure 5. Body weight change during the recovery period for active workers (AW) and rest-workers (RW). Data are shown as percentage change relative to baseline. Error bars indicate SEM. W4 indicates work day. R4–8 indicates recovery days 4 and 8. *** $p < 0.001$, compared to baseline; ## $p < 0.01$, between groups.

Active-workers gradually increased their body weight, and returned to baseline level in the course of the eight-day recovery period (Figure 5). On recovery day four, body weight was still significantly lower than baseline ($p < 0.001$), but not on recovery day eight ($p = 0.20$). Mean values of body temperature in the recovery period did not differ from baseline.

3.4. Metabolic Gene Expression in the Liver at the Shift in Feeding Rhythm of Rest-Workers

In our protocol, AW and RW animals were euthanized on opposite zeitgeber time points, which allowed for comparison to time-matched controls only. Transcriptional alterations in several key genes involved in glucose metabolism, insulin sensitivity, fatty acid synthesis, triglyceride synthesis, cholesterol synthesis, and fatty acid oxidation were present in RW compared to undisturbed, time-matched control animals (Figure 6). mRNA levels for the genes encoding Insulin receptor substrate 2 (*Irs2*), Hydroxymethylglutaryl-CoA synthase, cytoplasmic (*Hmgcs1*), and Glycerol-3-phosphate acyltransferase 1, mitochondrial (*Gpam*) were all upregulated in RW compared to time-matched controls. Less-pronounced upregulation of *Hmgcs1* was present in AW, compared to time-matched controls. Transcriptional levels of Fatty acid synthase (*Fas*) were upregulated four-fold both in RW and in AW, while transcription of Stearoyl-CoA desaturase 1 (*Scd1*) was significantly downregulated in the liver from both groups. Transcription of Peroxisome proliferator-activated

receptor alpha (*Ppara*, a major regulator of fatty acid oxidation) was downregulated in RW, but not in AW.

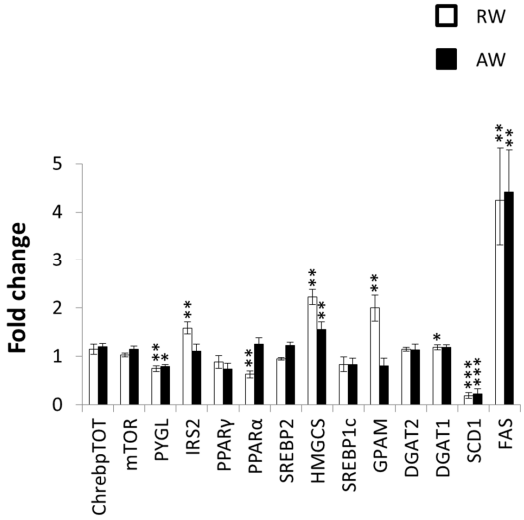

Figure 6. Expression levels of key metabolic genes in the liver from rest-workers (RW) and active workers (AW) following three work sessions, compared to undisturbed rats never exposed to simulated shift work. AW (n = 10) were sacrificed at zeitgeber time (ZT)24, and RW (n = 10) at ZT12. Undisturbed rats (RW control (n = 5) and AW control (n = 5) were sacrificed in a time-matched manner (i.e., at the same zeitgeber times as experimental animals). Fold changes for each gene represent the relative difference between shift work rats and controls. A fold change of 1 means no difference compared to controls, while a fold change of 2 means a doubling of transcriptional level, and a fold change of 0.5 a halving of the transcriptional level. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ChrebpTOT, Carbohydrate-responsive element-binding protein; mTOR, Machanistic target of repemycin; PYGL, Phosphorylase, glycogen, liver; IRS2, Insulin receptor substrate 2; PPAR, Peroxisome proliferator-activated receptor; SREBP, Sterol regulatory element-binding protein; HMGCS, Hydroxymethylglutaryl-CoA synthase; GPAM, Glycerol-3-phosphate acyltransferase 1 mitochondrial; DGAT, Diacylglycerol O-acyltransferase; SCD1, Stearoyl-CoA desaturase 1; FAS, fatty acid synthase.

4. Discussion

The aim of this study was to examine how one period of simulated night-shift work affects metabolism in male rats. Results show that three successive days, each with 8 h of forced activity, led to a shift in the feeding rhythm during the rest-work sessions in rats. Measures of food intake and body weight appear to partially stabilize after three days of rest-work, indicating an adaptation in the regulation of metabolism at this time point.

In this study, both RW and AW exhibited an overall reduction in food intake during the four-day work period. Other studies in animals report inconsistent results regarding food intake during day- and night-shift work. A review of animal models of shift work and effects on metabolism found that effects depended on the species examined (rat or mouse) and the protocol used (manipulate timing of activity, light, food intake, and/or sleep) [21]. In human night workers, a recent meta-analysis reported no change in total calorie intake [26], although there are many other documented changes, such as timing of food intake, number, size, and nutritional content of meals [27].

Our data demonstrated that the metabolic gene expression after three days of work was more affected in RW than in AW. Correspondingly, a previous study concluded that following a long

period (five weeks) of forced shift work, synchronization of clock genes (controlled by the SCN) and metabolic genes was lost in the liver of RW rats, primarily due to a shift in food intake towards the rest phase [28]. We found that the gene encoding *Irs2* (which is linked to liver insulin sensitivity and lipid metabolism) was upregulated in RW, in correspondence with reduced total food intake [29–31]. Upregulation of the gene encoding the central cholesterol-producing enzyme *Hmgcs1* could also be related to reduced food intake, with SREBP2 activation triggered by reduced sterol levels in the liver. However, the genes encoding *Fasn*—the rate-limiting enzyme in de novo fatty acid synthesis—and *Gpam*–which catalyses the first acylation step in the synthesis of glycerophospholipids—were also upregulated in RW. Such upregulation would not be expected to accompany decreased food intake [32]. The increased hepatic expression of these genes in RW may be indicative of paradoxical or compensatory fatty acid and triglyceride synthesis in the liver, via as-of-yet undisclosed molecular mechanisms. In addition, the reduced expression of *Ppara*—indicating decreased fatty acid oxidation—is paradoxical. This uncoupling between food intake, body weight, and lipid biosynthesis/oxidation underlines the metabolically-disruptive potential of rest-work.

While these data on liver gene expression clearly suggest effects of rest phase work, the results should be considered with some care, for several reasons. First, liver gene expression was measured at only a single time point after three work days in both the AW and RW groups. Since time of day was controlled for by comparing gene expression of working animals to undisturbed time-matched controls, the results do not reflect effects of zeitgeber time only, but more probably reflect the effects of forced activity. Nevertheless, a more detailed study is required to understand the temporal dynamics of these changes in gene expression, not only in the course of the four-day work period, but also during recovery thereafter. Second, in our current protocol, animals were fasted from 2 h before euthanasia, but this may not fully exclude the possibility that our gene expression data were affected by difference in food intake prior to tissue harvesting. Additionally, for this reason, future studies are required to assess in more detail the complex interactions between food intake, metabolic state, and gene expression in the liver. Moreover, in order to examine the significance of transcriptional alterations, relevant protein levels and metabolites such as glucose, insulin, and lipids should be investigated in future studies.

Very few animal studies report or discuss immediate early metabolic effects of night-shift work. Some studies present day-by-day body weight data visually, and appear to indicate a shift in metabolic (body weight) regulation following 2–3 days of simulated night-shift work, but this shift is generally not acknowledged or discussed [33–35]. Our data demonstrated that AW maintained a normal rhythm of food intake during the work period, consuming most of the food during their usual active phase, whereas RW gradually shifted timing of food intake towards working hours in their normal rest phase, with a stable high food intake reached and maintained at the third work session. A shift in timing of food intake is consistent with previous findings in the laboratory rat [36]. In parallel, human night-shift workers typically consume the majority of their calories during shifts [37]. Interestingly, the shift in food intake occurred between work days two and three in our study—a time window that parallels the shift in metabolism-related processes during simulated night-shift in humans [20,38].

In accordance with the overall reduction in food intake, a net weight loss was observed during the shift work period. Weight loss was more evident in RW than AW, and although body weight increased sharply during the eight-day recovery period, RW did not fully recover body weight to baseline level. Previous studies have reported both weight gain and weight loss during simulated rest-work [34,36]. However, in these studies, rats were exposed to much longer periods of rest-work (4–5 weeks), making it difficult to directly compare our short-term results. Additionally, in the present study, simulated work was achieved with wheels rotating at a faster pace than in previous studies (e.g., [34,36]), which might explain a more negative energy balance. Still, the rats in our study were forced to walk only approximately 1.1 km each work session. Although this physical activity is not considered strenuous, as rats with free access to a running wheel may voluntarily run 6–9 km each day [39], the work sessions may have contributed to weight loss through increased energy expenditure. Such

increased energy expenditure might be a direct consequence of the increased physical activity, but it might also partly depend on indirect mechanisms, such as an elevated body temperature. Intriguingly, this presumed increase in energy expenditure was not compensated for by food intake.

While the rats in the RW group consumed more food during the 8 h work sessions than they normally would during the same phase of the day under baseline conditions, they reduced food intake during the 16 h period between sessions (the net result being a reduction in 24-h food intake). Despite this reduction in energy intake between work sessions, body weight increased between sessions, presumably because the lower energy intake was compensated for by parallel reduction in energy expenditure. In agreement with this, RW body temperature was decreased between sessions, perhaps partly as a consequence of increased sleep [23]. This possibly represents a compensatory mechanism aimed at maintaining body weight. A previous study in humans suggests that two to three days of night-shift work is required for such metabolic compensatory mechanisms to take effect [20]. Thus, it may be that the initial days of night-shift work are the most straining on the metabolic system. Measurements of thermogenesis could have shed light on the energy expenditure gap demonstrated by our results, and should be included in future experiments.

Mean body temperature levels in AW rapidly normalized on the first day of recovery. Surprisingly, while RW initially recovered from the aberrant mean body temperature patterns displayed during the work period, they developed progressive hypothermia in the rest phase later on in the recovery phase. On recovery day eight, rest phase mean body temperature was almost 0.3 °C lower than at baseline. Since recordings were ended at recovery day eight, we do not know how many days hypothermia lasted, or how severe it became. The long-term effect on body temperature may be due to either stress, metabolic dysfunction, or a combination of both [40–43]. Based on the unexpected failure of all parameters to return to baseline levels, future studies should aim to prolong recovery time after forced work periods.

The model applied in the present study inherently entails that RW were exposed to light during work sessions, whereas AW were not. Due to the study design, we are precluded from investigating the contribution of light exposure to the metabolic changes. This should be addressed in future studies.

5. Conclusions

Our results indicate that in male rats, 3–4 days of forced work is sufficient to induce disruption to several metabolic parameters, particularly in rats exposed to forced activity during their rest phase. The current findings suggest that the initial days of night-shift work put the most strain on the metabolic system, inducing compensatory effects after 2–3 days, and that neither body weight nor body temperature is fully normalized in eight days of recovery.

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Author Contributions: Janne Grønli, Ståle Pallesen, Silje Skrede, Peter Meerlo and Jelena Mrdalj conceived and designed the experiments; Janne Grønli, Jelena Mrdalj, Andrea Rørvik Marti, Torhild Thue Pedersen and Silje Skrede performed the experiments; Janne Grønli, Jelena Marti, Sjoerd Johan van Hasselt and Silje Skrede analysed the data; Andrea Rørvik Marti, Peter Meerlo, Janne Grønli, Sjoerd Johan van Hasselt, Jelena Mrdalj, Ståle Pallesen, Torhild Thue Pedersen, Tone Elise Gjøtterud Henriksen and Silje Skrede wrote the paper.

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No Escaping the Rat Race: Simulated Night Shift Work Alters the Time-of-Day Variation in BMAL1 Translational Activity in the Prefrontal Cortex

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Millions of people worldwide work during the night, resulting in disturbed circadian rhythms and sleep loss. This may cause deficits in cognitive functions, impaired alertness and increased risk of errors and accidents. Disturbed circadian rhythmicity resulting from night shift work could impair brain function and cognition through disrupted synthesis of proteins involved in synaptic plasticity and neuronal function. Recently, the circadian transcription factor brain-and-muscle arnt-like protein 1 (BMAL1) has been identified as a promoter of mRNA translation initiation, the most highly regulated step in protein synthesis, through binding to the mRNA "cap". In this study we investigated the effects of simulated shift work on protein synthesis markers. Male rats (n = 40) were exposed to forced activity, either in their rest phase (simulated night shift work) or in their active phase (simulated day shift work) for 3 days. Following the third work shift, experimental animals and time-matched undisturbed controls were euthanized (rest work at ZT12; active work at ZT0). Tissue lysates from two brain regions (prefrontal cortex, PFC and hippocampus) implicated in cognition and sleep loss, were analyzed with m^7GTP (cap) pull-down to examine time-of-day variation and effects of simulated shift work on cap-bound protein translation. The results show time-of-day variation of protein synthesis markers in PFC, with increased protein synthesis at ZT12. In the hippocampus there was little difference between ZT0 and ZT12. Active phase work did not induce statistically significant changes in protein synthesis markers at ZT0 compared to time-matched undisturbed controls. Rest work, however, resulted in distinct brain-region specific changes of protein synthesis markers compared to time-matched controls at ZT12. While no changes were observed in the hippocampus, phosphorylation of cap-bound BMAL1 and its regulator S6 kinase beta-1 (S6K1) was significantly reduced in the PFC, together with significant reduction in the synaptic plasticity associated protein activity-regulated cytoskeleton-associated protein (Arc). Our results indicate considerable time-of-day and brain-region specific variation in cap-dependent translation initiation. We conclude

that simulated night shift work in rats disrupts the pathways regulating the circadian component of the translation of mRNA in the PFC, and that this may partly explain impaired waking function during night shift work.

Keywords: circadian rhythms, sleep deprivation, cognition, synaptic plasticity, protein synthesis, eIF4E, BMAL1, arc

INTRODUCTION

Millions of people worldwide work at times that overlap with the normal time for sleep, resulting in significant cognitive impairment and somatic symptoms (Rajaratnam and Arendt, 2001). In humans, night shift work in both real-life and laboratory simulations, is known to induce circadian rhythm disturbance which has been linked to deficits in cognitive functions (Folkard, 2008; Veddeng et al., 2014; Maltese et al., 2016; Pilcher et al., 2016). Night workers often report impaired alertness and performance on duty, and the risk of errors and accidents is increased at night (Folkard and Tucker, 2003; Åkerstedt et al., 2010; Øyane et al., 2013; Kazemi et al., 2016).

Sleep is regulated by both a homeostatic regulated sleep propensity process that builds up across hours of wakefulness, and an independent circadian process that oscillates with a period of about 24 h. In humans, the circadian factor typically promotes sleep at night and wakefulness during the day (Borbély, 1982). Hence, night shift work induces a mismatch between work demands and the brain's state promoting sleep due to the homeostatic and circadian sleep drive (Gupta and Pati, 1994; Flo et al., 2012). It is conceivable that night work and other forms of circadian rhythm disruption decrease alertness and cognitive functioning through disruption of processes involved in regulating synaptic strength and neuronal communication. Plasticity at the level of the synapse represents a set of dynamic changes in the strength of information transfer between neurons, considered essential for processing and storage of information in the brain, and for the ability to adapt to and learn from the external environment. Many of the molecular events associated with long-term modification of synaptic strength are subject to circadian regulation (Gerstner and Yin, 2010), including synapse to nucleus signaling, neuronal activity-dependent gene transcription and protein synthesis.

Protein synthesis, synaptic plasticity and circadian rhythmicity are fundamental properties of the brain and are tightly regulated to environmental and cellular changes. The initiation step of mRNA translation is the most highly regulated step of protein synthesis (Siddiqui and Sonenberg, 2015; Bramham et al., 2016; **Figure 1**). Recently, the circadian clock has been linked to translation initiation processes (Lipton et al., 2015). In its phosphorylated form, the well-known circadian transcription factor brain-and-muscle arnt-like protein 1 (BMAL1) promotes cap-dependent translation (**Figure 1A**). The mTOR/S6 kinase beta-1 (S6K1) pathway is a critical regulator for phosphorylation of BMAL1 necessary to facilitate both association with the translational machinery and stimulation of protein synthesis (Lipton et al., 2015). In addition to several proteins that promote translation in neurons, there are also proteins that repress translation initiation (Napoli et al., 2008; Bidinosti et al., 2010; Gkogkas et al., 2010; Gal-Ben-Ari et al., 2012; Kong and Lasko, 2012; De Rubeis et al., 2013; **Figure 1B**).

A number of other cellular components are in addition involved in mediating protein synthesis-dependent synaptic plasticity. One regulator of synaptic activation is the activity-regulated cytoskeleton-associated protein (Arc). Arc acts as a multifunctional activity-induced hub protein important for mediating and organizing long-term synaptic plasticity, with impact on learning, memory and behavior (Guzowski et al., 2000; Kelly and Deadwyler, 2003; Bramham et al., 2010; Nikolaienko et al., 2017). Arc regulation has been implicated in sleep, wakefulness and in circadian rhythm regulation (Nishimura et al., 2003; Cirelli et al., 2004; Thompson et al., 2010).

We have recently established a rat model of shift work, where rats are exposed to enforced ambulation in slowly rotating wheels for 8 h/day, either in their rest phase ("rest work"; to simulate night shift work) or in their active phase ("active work"; to simulate day shift work; Marti et al., 2016; Grønli et al., 2017). Using this model, we observed a progressive intrusion of spontaneous cortical slow waves and micro-sleeps during rest work across four consecutive days, which was not observed during active work (Grønli et al., 2017). Similar findings have been observed in human studies, and likely contribute to decreased alertness during the night shift (Torsvall and Åkerstedt, 1987; Torsvall et al., 1989). Importantly, in our study, the degraded waking state during rest work could not be explained by sleep loss alone. Across the 16 h opportunity to sleep after work, the total amount of sleep was not different between rest and active workers.

Considering the importance of protein synthesis for activity-dependent synaptic plasticity and homeostatic plasticity (synaptic scaling), and the role of the circadian protein BMAL1 in translation initiation, it is conceivable that disruption of translation initiation may contribute to the impaired waking function observed in night shift workers. Sleep is associated with enhanced rates of protein synthesis (Cirelli et al., 2004), and sleep deprivation has been shown to have differential effects on the protein synthesis in the hippocampus and the PFC, respectively (for review see Grønli et al., 2014). Overall, insufficient sleep negatively affects protein translation, but sleep of good quality prior to sleep restriction may diminish these negative effects (Grønli et al., 2012). However, the effects of degraded waking function, or of simulated night shift work, on protein synthesis have not yet been considered. Moreover, it is

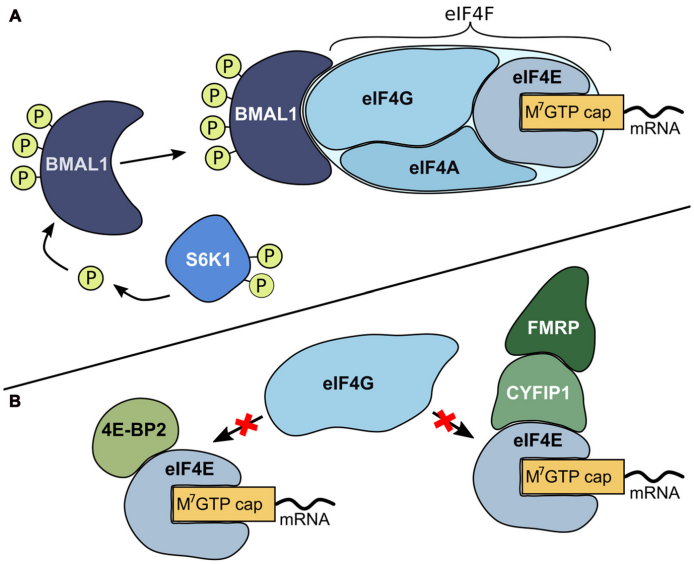

FIGURE 1 | Mechanisms for the regulation of translation initiation. **(A)** Promotion of translation. Initiation starts with the formation of the eukaryotic initiation complex 4F (eIF4F). This complex consists of the translation initiation promoting factor eIF4E that binds to the mRNA m^7GTP (cap) structure. Subsequently, eIF4E recruits cap-binding proteins like the scaffolding protein eIF4G and the RNA helicase eIF4A and form a multi-protein translation initiation complex. In its phosphorylated form, the circadian transcription factor brain-and-muscle arnt-like protein 1 (BMAL1) is capable of binding to the eIF4F complex and functions as a translation factor to further enhance translation. Ribosomal protein S6 kinase beta-1 (S6K1), an mTOR derived kinase, is a critical regulator for phosphorylation of BMAL1 (Lipton et al., 2015). **(B)** Repression of translation. There are several proteins that repress translation initiation. eIF4E-binding proteins (4E-BPs, for example 4E-BP2), cytoplasmic fragile X mental retardation-interacting protein (CYFIP1) and its binding partner fragile X mental retardation protein (FMRP) bind to eIF4E. When these proteins are bound to eIF4E, eIF4G cannot bind and hence eIF4F complex formation is prevented (Napoli et al., 2008; Bidinosti et al., 2010; Gkogkas et al., 2010; Gal-Ben-Ari et al., 2012; Kong and Lasko, 2012; De Rubeis et al., 2013).

still not known how other proteins involved in cap-dependent translation are regulated at different times of day and across different brain regions, or how simulated shift work affects these dynamics.

In the present study, we decided to use the cap-pulldown technique to determine the time-of-day and brain region specific variation in cap-dependent mRNA translation. Cap-pulldown is a valuable tool for examining the processes that are occurring directly on the mRNA cap, regulating the initiation of translation. It was decided to first characterize the time-of-day dependent variation in the expression of cap-associated proteins important for promoting translation initiation and repressing translation initiation, and regulation of synaptic plasticity. We hypothesized that cap-bound BMAL1, and its regulator S6K1, has specific time-of-day (lights ON vs. lights OFF) expression in two brain regions; the prefrontal cortex (PFC) and hippocampus, as these regions are implicated in cognition and are vulnerable to disturbance of circadian rhythms and sleep loss (Brown and Bowman, 2002; Cirelli et al., 2004; Gerstner and Yin, 2010; Karatsoreos et al., 2011; Alberca-Reina et al., 2015). Second, we wanted to investigate how these dynamics are affected by three consecutive days of simulated shift work.

MATERIALS AND METHODS

Ethical Approval

This study was carried out in accordance with Norwegian laws and regulations, and The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The protocol was approved by the Norwegian Animal Research Authority (permit number: 2012463).

Animals and Housing

Two batches of 40 male rats in total (n = 24 Wistar, nTach:WH; n = 16 Sprague-Dawley nTac:SD; Taconic, Silkeborg, Denmark) weighing approximately 300 g at arrival, were used in the study. Different rat strains were chosen because the supplier (Taconic) no longer deliver the Wistar strain. The procedures were otherwise the same for both experiments. All animals were group housed in individually ventilated cages (IVC, Techniplast, Buggugitate, Italy, 75 air changes/h) type IV (480 × 375 × 210 mm, 1500 cm^2). The animals were maintained on a 12 h light/12 h dark (LD) schedule with lights on at 06:00 (zeitgeber time 0; ZT0). Lights were gradually dimmed on and

off over a period of 1 h (fully on at 07:00 and fully off at 19:00). Filtered water and food were available *ad libitum* throughout the experiment (rat and mouse No. 1, Special Diets Services, Witham, Essex, UK). During the experimental protocol, all animals were single housed (IVC cage type III, 425 × 266 × 185 mm, 800 cm^2).

Experimental Protocol

To simulate shift work, animals were exposed to forced activity for 8 h per day, centered either in the rats' normal active phase (active work; ZT14–22; n = 10) or in the rats' normal rest phase (rest work; ZT2–10, n = 10). Animals were placed in automatically rotating wheels (Rat Running Wheel, TSE running wheel system, Bad Homburg, Germany; 24 cm diameter; 3 rpm; 1440 revolutions or 1.086 km of linear distance per 8 h session). Food and water was available *ad libitum*. Rotating wheel, feeders and water bottles were cleaned after each work session with 5% ethanol solution. Between sessions, animals were housed in their home cage. Work schedules were repeated for 3 days.

Tissue Collection

Following the third work session, animals were placed in their home cage for 2 h. Subsequently, they were anesthetized with isoflurane, and sacrificed by decapitation. Active workers were sacrificed at ZT0, at lights ON, and rest workers at ZT12, at lights OFF. A separate group of undisturbed animals never exposed to simulated work were used to investigate time-point specific protein variation, and sacrificed at the same times as experimental animals (ZT0, at lights ON, n = 10; and ZT12, at lights OFF, n = 10).

m^7GTP (Cap) Pull-Down

m^7GTP pull-down assays have been described in detail elsewhere (Panja et al., 2014). Bilateral hippocampus and PFC were separately homogenized in 1000 µl of m^7GTP lysis buffer (50 mM Tris, 100 mM NaCL, 1 mM EDTA, NP-40 0.5%, 1 mM dithiothreitol, 1 mM Na_3VO_4, 50 mM NaF, and 1× protease inhibitor cocktail from Roche). The homogenate was centrifuged 10 min at 14,000 g at 4°C. For the m^7GTP pull down, 300–400 µg of protein together with 30 µl of 7-methyl GTP-agarose beads (Jena bioscience #AC-141) were incubated 2 h at 4°C. Beads were washed three times with m^7GTP lysis buffer and bound proteins were separated to an SDS-PAGE (10% gels). Immunoblotting was carried out as described above.

SDS-PAGE and Immunoblotting

Antibodies used for immunoblotting were as follows: p-eIF4E (1:1000, Cell Signaling #9741), eIF4E (1:1000, Cell Signaling #9742), eIF4G (1:1000, Cell Signaling #2498), p-BMAL1 (1:1000, Cell Signaling #13936), total BMAL1 (1:500; Santa Cruz Biotechnology #sc365645), p-pS6k (1:1000, Santa Cruz Biotechnology #sc-7984), pS6k (1:1000, Sigma #SAB4502691), 4E-BP2 (1:1000, Cell Signaling #2845), CYFIP1 (1:1000, Upstate #07-531), fragile X mental retardation protein (FMRP; 1:1000, Abcam #17722), Arc (1:500; Santa Cruz Biotechnology #sc17839), and GAPDH (1:5000, Santa Cruz Biotechnology #sc32233).

Samples from cap pull-down assays and lysates were boiled in laemmli sample buffer (Bio-Rad) and resolved in 10% SDS/PAGE gels. Proteins were transferred to nitrocellulose membranes (Biorad, #162-0112) which were then blocked with 5% non-fat dry milk, probed with antibodies and developed using chemiluminescence reagents (Pierce, #32106). The blots were scanned using Gel DOC XRS+ (BIO RAD) and densitometric analyses were performed with ImageJ software (NIH, Bethesda, MD, USA). Blots treated with phospho-specific antibody stripped with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl, pH 6.8 at 50°C for 30 min, washed, blocked and reprobed with antibody recognizing total protein. Densitometric values expressed per unit of protein (tot-eIF4E/GAPDH as specified) applied to the gel lane. The cap-pulldown was conducted in order to assess changes in the association of eIF4E binding proteins with eIF4E. For this analysis the amount of eIF4G, p-BMAL1, total-BMAL1, CYFIP1, FMRP and 4E-BP2 was normalized to the total amount of recovered eIF4E. The phospho-proteins were normalized relative to the total protein on the same lane. Total proteins were normalized to loading control.

Statistical Analyses

Significant effects of the time-of-day, work conditions, brain regions and experiments were determined using 2 × 2 factorial analysis of covariance (ANCOVA), with "time-of-day" or work condition (2 levels) and "brain region" (2 levels) as independent variables, and experiment as covariate. Different degrees of freedom in the results section are due to exclusion of some animals from analysis when showing values >2 standard deviations from the group mean. Additionally, one sample was lost due to a technical issue (ZT12 control, PFC). The p-value for significance was 0.05. Fisher's LSD was applied as *post hoc* test. Cohen's d was calculated as measure of effect size. As a mean for interpreting d, 0.2 is considered small, 0.5 medium and 0.8 large effect size, respectively (Cohen, 1988).

RESULTS

Changes in protein were assessed in homogenate samples obtained from micro-dissected PFC and hippocampus. Proteins actively bound to the cap-structure (cap-bound) and total concentrations of individual proteins available in the sample (input data) were measured. All statistical results are provided in Supplementary Tables S1–S6, and Supplementary Figures S1, S2 illustrate the results on input data.

Time-of-Day Specific Variation in Different Brain-Regions

We first examined expression levels of cap-bound promoters of translation. In PFC, two key translational promoters, the translation initiation factor p-eIF4E and the circadian transcription factor p-BMAL1 exhibited a significant effect of time-of-day, with significant increase of cap-bound phosphorylation at ZT12 (lights OFF) compared to ZT0

(lights ON; p-eIF4E: +57.7%, $p = 0.03$, $d = 1.44$; p-BMAL1: +143.4% at ZT12, $p < 0.001$, $d = 1.57$). This effect was not evident in the hippocampus (**Figures 2A,B**, immunoblots **Figure 2I**). The eIF4G, a scaffolding protein critical for formation of the translation initiation complex, and tot-BMAL1 (both phosphorylated and unphosphorylated BMAL1) showed no significant time-of-day effect, neither in the PFC nor in the hippocampus. Analysis of tissue lysate (inputs) suggests that all promoters were similarly expressed across both time points and brain regions (Supplementary Figures S1A,B).

Critical for BMAL1 to interact with the translational machinery is the regulator S6K1, as Lipton et al. (2015) showed that p-S6K1 phosphorylates BMAL1. In parallel with increased p-BMAL1 in PFC, p-S6K1 showed significantly higher expression at ZT12 compared to ZT0 (+118.1%, $p = 0.002$, $d = 1.08$). There was no significant effect of time-of-day in the hippocampus (+18.0%, $d = 0.29$; **Figures 2C,D**, immunoblots **Figure 2J**).

There were no significant time-of-day effects of the cap-bound translational repressors 4E-BP2, CYFIP1 and its binding partner FMRP (**Figures 2E,F**, immunoblots **Figure 2I**). Input data showed that total protein for all repressors were similarly available in all tissues and time points (Supplementary Figures S1C,D, Supplementary Table S4).

In PFC, the expression of Arc protein, a key regulator of long-term synaptic plasticity and synaptic scaling (Bramham et al., 2008, 2010; Korb and Finkbeiner, 2011; Shepherd and Bear, 2011) was decreased at ZT12 compared to ZT0 (−42.1%, $p = 0.001$, $d = 1.13$). There was no significant effect of time-of-day in the hippocampus (+18.3%, $p = 0.68$, $d = 0.30$; **Figures 2G,H**, immunoblots **Figure 2J**).

Overall, these results conform to findings by Lipton et al. (2015), suggesting that together with the known translational initiation promoter eIF4E, translation is linked to circadian timing by cap-bound p-BMAL1 at ZT12. Furthermore, the parallel increase in phosphorylated S6K1 at ZT12 supports

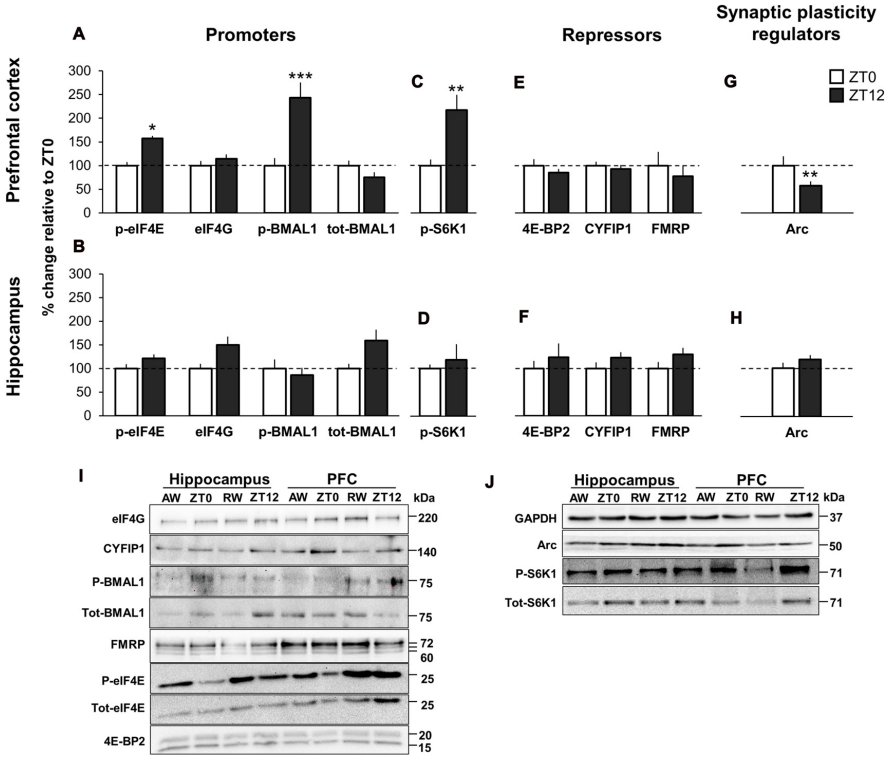

FIGURE 2 | Time-of-day variation in promoters and repressors of cap-dependent translation initiation, and synaptic plasticity regulators in undisturbed animals. **(A,E)** m^7GTP pull-down analysis of prefrontal cortex (PFC) and **(B,F)** hippocampus lysates. **(C,G)** Western blot analysis of PFC and **(D,H)** hippocampus lysates. Brain tissue was collected at lights ON (zeitgeber time 0, ZT0) and at lights OFF (ZT12). Quantification of immunoblot is expressed as percentage change relative to ZT0 (normalized to 100%). Error bars represent SEM. Significant differences: $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **(I)** Representative immunoblot for **(A,B,E,F)**. Blots normalized to total eIF4E in the corresponding immunoblot. **(J)** Representative immunoblot for **(C,D,G,H)**. Blots normalized to GAPDH in the corresponding immunoblot.

Lipton et al. (2015) conclusions that BMAL1 is a substrate of S6K1. However, our data suggests that this is only the case in PFC and not in the hippocampus. Additionally, the present data suggest a differential role of Arc depending on time-of-day and brain region.

Effects of Shift Work

To investigate the effects of three consecutive days of simulated shift work in either the normal active phase ("active work"; simulated day shift work) or the normal rest phase ("rest work"; simulated night shift work), changes in the translational

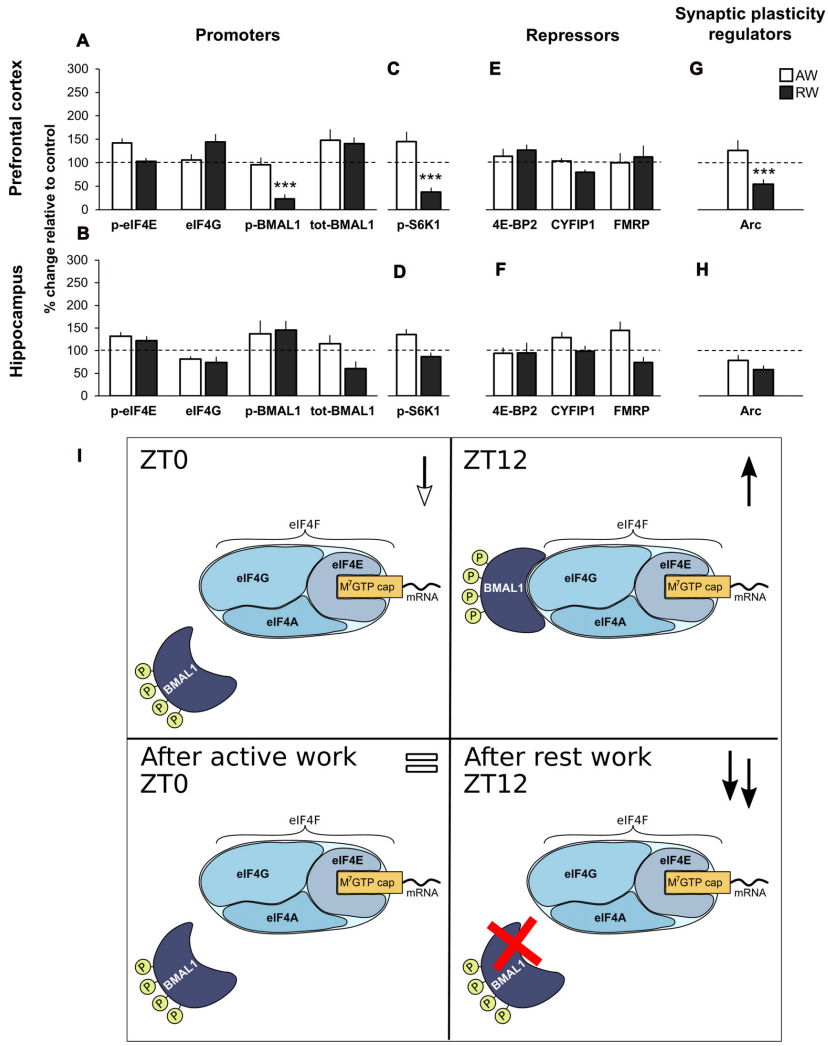

FIGURE 3 | Effect of simulated shift work on promoters and repressors of cap-dependent translation initiation, and synaptic plasticity regulators relative to undisturbed controls. **(A,E)** m^7GTP pull-down analysis of PFC and **(B,F)** hippocampus lysates. **(C,G)** Western blot analysis of PFC and **(D,H)** hippocampus lysates. Rats were exposed to forced activity during the active phase (AW, active work; brain tissue was collected at ZT0) or during the rest phase (RW, rest work; brain tissue was collected at ZT12). Quantification of immunoblot is expressed as percentage change relative to time-matched undisturbed control (normalized to 100%). Error bars represent SEM. Significant differences: $^{***}p < 0.001$. For representative immunoblot see **Figure 2**. **(I)** Schematic model of promotion of translation initiation in undisturbed animals (ZT0 and ZT12), and following simulated shift work (active work and rest work). ↓ decreased expression compared to ZT12; ↑ increased expression compared to ZT0; = similar expression compared to ZT0; ↑↑ increased expression compared to ZT12.

regulators in the two groups were each compared to respective time-matched undisturbed controls (active work at ZT0; rest work at ZT12).

Active Work Does Not Significantly Affect Cap-Bound Translation Initiation

There was no significant effect of active work on the expression of the translational promoters (p-eIF4E, eIF4G, p-BMAL1 and tot-BMAL1), neither for the cap-bound proteins, the input proteins, nor for the expression of phosphorylated S6K1. Effect sizes were small (>0.5) for all proteins measured, with the exception of p-eIF4E and p-S6K1 in PFC (+25.7%; d = 0.53 and +44.9%, d = 0.55, respectively). This is illustrated in **Figures 3A–D** (immunoblots **Figure 2I**).

There was also no significant change after active work on the expression of the repressors (4E-BP2, CYFIP1 and FMRP, cap-bound and input) or the expression of Arc (**Figures 3E–H**, immunoblots **Figures 2I,J**).

Input data showed that all promoters and repressor proteins were available similarly across groups (active work and time-matched control; Supplementary Figures S2A–D, immunoblots Supplementary Figure S1E).

These data indicate that 3 days of simulated day work does not significantly alter regulators of translation initiation, neither in the PFC nor hippocampus.

Rest Work Impairs Regulation of Translation Initiation in PFC by Reducing Cap-Bound p-BMAl1, p-S6K1 and Arc

Following rest work, the circadian translational promoter p-BMAL1 was significantly reduced in PFC (−77.0%, $p < 0.001$, d = 1.97), as was its regulator p-S6K1 (−62.4%, $p < 0.001$, d = 1.29). There was no significant effect of rest work in terms of the expression of cap-bound phosphorylated eIF4E and eIF4G (**Figures 3A–D**). There was no significant effect of rest work in the hippocampus.

No significant effect of rest work was found on the expression of the repressors (4E-BP2, CYFIP1 and FMRP, cap-bound and input; **Figures 3E,F**).

Input data showed that all promoters and repressor proteins were available similarly across groups (rest work and time-matched control) except for a significant increase in available p-eIF4E in the hippocampus following rest work (+107.2%, p = 0.004, d = 1.43; Supplementary Figures S2A–D, immunoblots Supplementary Figure S1E).

The synaptic plasticity regulator Arc was significantly reduced in PFC following rest work (−46.5%, $p < 0.001$, d = 1.49; **Figures 3G,H**).

According to our data, phosphorylated BMAL1 and p-S6K1 are normally higher in the PFC at ZT12 compared to ZT0. The present results showing a reduced p-BMAL1 and p-S6K1 after simulated night shift work demonstrate an impaired circadian promotion of translation initiation in PFC (**Figure 3I**).

Effects of Experiment

Data included in the analyses was pooled from two separate experiments on separate rat strains, as described in the method section. Thus, ANCOVA with experiment as covariate was run to examine whether any significant differences would occur between experiments. Overall, there were no significant effects of experiment on the translational promoters eIF4G and p-BMAL, both cap-bound and input. An effect of experiment was present in some analyses on translational repressors but the variation between experiments did not affect the variation across time-of-day, groups or brain tissue (Supplementary Tables S1–S6). Considering the overall uniformity of the data across experiments, and that our main findings on p-BMAL1 was upheld across rat strains, these findings suggest that the changes in protein expression reflect functionally meaningful impacts of time-of-day and rest work.

DISCUSSION

The aim of the present study was to examine time-of-day and brain-region specific variation in cap-dependent translation initiation in undisturbed rats and in rats exposed to simulated shift work. Decreased alertness, cognitive performance and quality of wakefulness has been observed in both humans and animals following night shift work, however, the neurochemical basis for these disruptions had yet to be investigated.

We found time-of-day specific differences in cap-bound protein expression and phosphorylation state in the PFC, in both undisturbed rats and rats exposed to simulated shift work. In undisturbed control rats, an upregulation of translational promoters was observed at the end of the resting phase (ZT12) as compared to the end of the active phase (ZT0). No significant up- or downregulation of translation initiation processes in the hippocampus were observed at ZT12 compared to ZT0.

Forcing rats to work during their normal active phase (active work) only resulted in non-significant changes in markers of protein synthesis regulation compared to time-matched controls. In contrast, a 3-day protocol of forced work during the normal resting phase (rest work) resulted in decreased phosphorylation of the circadian clock protein and translational promoter BMAL1 and its regulator S6K1, as well as decreased expression of the synaptic plasticity associated protein Arc in PFC. There were no significant effects of either active work or rest work on translation regulators in hippocampus. These results indicate that while the pathways regulating the circadian component of protein synthesis in the hippocampus appears to be spared, a disruption in this pathway in the PFC may underlie the cognitive deficits observed during night shift work.

Cap-Dependent Translation Initiation in Undisturbed Animals

The cap-pulldown technique is a valuable tool for examining changes in translation initiation activity, based on altered binding of translation factors to the 5′-cap structure and associated eIF4E. Previously, Lipton et al. (2015) used cap-pulldowns in mouse hepatic tissue to show diurnal regulation of translation mediated by phosphorylation of BMAL1, with binding of p-BMAL peaking during the active phase. The present study demonstrates time-of-day dependent variation in cap-bound regulators of translation in the brain. Within the PFC of undisturbed animals, we

observed that the cap-bound translational promoters p-eIF4E and p-BMAL1 were significantly increased at ZT12 compared to ZT0. These results indicate that cap-bound p-BMAL1 is actively up-regulating translation initiation at ZT12 in PFC. Notably, the variation in the PFC was evident only in the cap-pulldown analysis, and not in terms of input protein. This indicates that diurnal changes in the abundance of specific translation factors on the cap is due to changes in binding activity, and not to changes in levels of translation factor expression. The result underscores the importance of examining the dynamics of translation factor binding, which is not revealed by immunoblotting of tissue lysates.

No significant up- or downregulation of cap-dependent translation were observed in hippocampus at ZT12 compared to ZT0. Diurnal variation in p-eIF4E in hippocampal lysates (peak at ZT6, and no change between ZT12 and ZT0) has been reported (Saraf et al., 2014), however changes in cap- binding were not measured in that study. Another study examined expression of input protein in the hippocampal proteome and found that 1.7% of proteins showed diurnal variation (Chiang et al., 2017). This does not amount to a large proportion, but among proteins showing circadian variation were several of those found in the mTOR signaling pathway, which regulates BMAL1 phosphorylation through S6K1 (Lipton et al., 2015; Chiang et al., 2017). In liver and brain cells *in vitro*, cytoplasmic BMAL1 regulates translation initiation through the mTOR complex (Lipton et al., 2015). Our results in PFC support this, as the increase in p-BMAL1 was mirrored by an increase in the mTOR-derived regulator p-S6K1.

The role of BMAL1 as a transcription factor in the cell nucleus, where it is involved in clock gene regulation, has been well-characterized (for review see Albrecht, 2012). However, the role of cytoplasmic BMAL1 protein seems to be far separated from its transcriptional role. In fact, mTOR is increasingly standing out as a key regulator of cytoplasmic BMAL1 function. Over-activation of mTOR through knockout of the tuberous sclerosis (TSC) gene in mice causes an increase in cytoplasmic BMAL1 protein, as well as decrease in BMAL1 degradation, without affecting transcription of BMAL1 (Lipton et al., 2017).

The role of BMAL1 and BMAL1-interacting proteins in the cytoplasm still remains largely uncharacterized (Lipton et al., 2015). Only a few studies have started to identify the role of BMAL1 as a cap-dependent promoter of translation. Our results demonstrate that time- and tissue-specific variations exist in cap-dependent translation, and that there is still much to elucidate about the role of BMAL1.

Effect of Simulated Shift Work on Cap-Dependent Translation Initiation

Following rest work, we observed a reduction in cap-bound p-BMAL1 as well as its mTOR-stimulated regulator p-S6K1 in PFC. Importantly, we only observed changes to phosphorylation status of cap-bound BMAL1. There were no significant changes in cap-bound tot-BMAL1 or BMAL1 available as input protein. Thus, the observed changes seem to be directly related to dysregulation of the mTOR/S6K1 pathway in the PFC. These results suggest that the PFC may be particularly vulnerable to rest work. Although effect on PFC functioning after simulated night shift work has yet to be examined, other rodent models support that the PFC may be a brain region particularly vulnerable to disruption of circadian rhythms. In one study, exposure to a 10 h light/10 h dark cycle decreased neuronal complexity in the prelimbic PFC and impaired performance on the Morris water maze in mice (Karatsoreos et al., 2011). Importantly, reduction in performance was observed only when the task was sufficiently demanding and requiring cognitive flexibility, suggesting an important role for the PFC in this task.

Our data suggests that the mTOR/S6K1 pathway in the PFC is particularly vulnerable to night shift work at ZT12. Interestingly, both increased and decreased activity in mTOR signaling pathways have been shown to have detrimental effects on learning and memory processes (Lipton and Sahin, 2014). Two models reflecting over- and under- activity of mTOR (TSC mice and S6K1 knock out mice, respectively) both show deficits in the early stage of long-term potentiation (LTP) formation and in the acquisition of the Morris water maze test (Goorden et al., 2007; Antion et al., 2008). Additionally, expression of dominant-negative S6K1 mutant in rat medial PFC resulted in less active coping behavior in the forced swim test (Dwyer et al., 2015). It is tempting to speculate that disruption of these processes may contribute to the degraded waking state observed in rats exposed to simulated night shift work (Grønli et al., 2017).

Rest work did not impair the mTOR/S6K1-derived pathway regulating translation in hippocampus in the present study. Previous studies show mixed results when it comes to the impact of simulated night shift work or similar interventions on hippocampal functions. In one study, 5 weeks of rest work did not impair performance on a hippocampus-dependent instrumental learning task (Leenaars et al., 2012). It is possible that the hippocampus is more vulnerable to sleep loss than to circadian rhythm disturbance. The effects of sleep amount and quality on hippocampal cellular and molecular processes, critical for learning, memory and spatial navigation, have been well characterized (Meerlo et al., 2009; Prince and Abel, 2013; Havekes and Abel, 2017). In our model, total sleep time was reduced similarly in rest workers and active workers (11%–12% compared to baseline), and cumulative slow-wave energy during non-REM sleep did not differ between the groups (Grønli et al., 2017). Still, slow wave intrusion during work was observed in rest workers only. Moreover, mathematical modeling of the sleep state dynamics demonstrated that differences in slow-wave-sleep bout duration between active and rest work groups largely reflected a circadian effect (Rempe et al, unpublished data). Chronic phase shifting does disrupt hippocampal function (e.g., Craig and McDonald, 2008), and also causes a 10% reduction in sleep time in mice (Brager et al., 2013). If the hippocampus is sensitive to sleep deprivation specifically, our model may not have sufficiently sleep deprived the animals, and thus did not induce significant hippocampal effects.

Sleep loss due to forced activity may be affecting the neuroendocrine systems by altering the state or function of

the hypothalamo-pituitary-adrenal (HPA) axis. Both the PFC and the hippocampus are brain regions sensitive to secretion of corticosterone. The hippocampus has a high concentration of glucocorticoid receptors and hence increased sensitivity to glucocorticoids as this brain region is involved in the negative feedback response to release of glucocorticoids. Corticosterone can affect clock gene regulation and synaptic function (Takahashi et al., 2013; Al-Safadi et al., 2014), thus we cannot rule out that the effects observed may be caused by increased activity in the HPA axis as a result of forced activity in the rest phase. However, since the present study demonstrates effects on translational modulators and markers of synaptic plasticity in PFC and not hippocampus, this suggests that simple effects of corticosterone alone are likely not an essential driver of the observed effects.

The Synaptic Plasticity Regulator Arc; Diurnal Variation and Sleep

The role of Arc in synaptic plasticity and learning has been extensively studied, but its diurnal variation has been less examined. Arc expression has been shown to differ between time-points (night vs. day) in pineal cells (Yeung Lam et al., 2004), and is involved in circadian phase-shift in response to light in the SCN (Nishimura et al., 2003). In PFC, Arc mRNA shows diurnal variation, with higher expression at ZT1 vs. ZT11, indicating that transcription of Arc is regulated by a diurnal component in this brain structure (Calabrese et al., 2011).

Arc mRNA transcription typically increases during the active phase and decreases during the rest phase, throughout the cortex (Cirelli and Tononi, 1999; Cirelli et al., 2004; Grønli et al., 2014). However, translation of Arc protein does not necessarily follow the changes in transcription. In the present study, we showed that Arc protein was reduced at the end of rest phase (ZT12) relative to the end of active phase (ZT0) in PFC. These data mimic previous findings by Thompson et al. (2010). However, the present findings also indicate that Arc protein shows a brain-region specific expression. Arc protein was expressed in an opposite pattern in the PFC (highest at ZT0) compared to the hippocampus (highest at ZT12). Another study showed that Arc translation was in fact increased in the cat visual cortex during the early hours of sleep (2 h after sleep onset; Seibt et al., 2012). In a previous study, we showed that, while Arc mRNA was increased in PFC after 8 h sleep deprivation, Arc protein was not (Grønli et al., 2012). Thus, Arc protein expression appears to be dynamically regulated in a tissue-dependent way both by time-of-day, and by sleep and wake states (Grønli et al., 2014).

The effects of simulated shift work or disturbance of circadian rhythms on the dynamics of Arc expression, to our knowledge have not been investigated previously. The present study demonstrates that simulated night shift work in rats significantly reduced expression of Arc protein in the PFC at ZT12. Arc protein is most recognized as an activity-induced protein, and not commonly known to decrease from baseline levels. We believe this is an effect reflecting circadian disturbance, and not an effect of reduced sleep as the 8 h forced ambulation increases the daily time awake and reduces the daily time in sleep equally in both rest- and active workers (Grønli et al., 2017). The only difference between rest- and active workers was the time at which the simulated shift work occurred. Our results on reduced expression of Arc protein in the PFC following rest work further indicate that the PFC may be more vulnerable than the hippocampus to the effects of night shift work.

Implications

The present work has provided a deeper understanding of the mechanisms that underlie the impact of shift work on protein synthesis, important for long-term modification of synaptic strength and the brain's ability to adapt to and learn from the external environment. The present results suggest that the degraded wake functioning observed in both humans and animals, as well as in both field and laboratory studies, may derive from reduced synaptic efficiency in the PFC. The PFC is crucial for higher-order cognition and complex tasks, such as attention and decision making (Brown and Bowman, 2002). It was suggested already in 1993 that the PFC may be particularly vulnerable to the effects of sleep loss (Horne, 1993; Alhola and Polo-Kantola, 2007). We suggest that this vulnerability may not only be due to sleep loss, but rather due to a mismatch between work demands and the brain's ability to overcome the homeostatic and circadian challenges imposed by night shift work. Future animal studies should combine behavioral testing, measures of wake functioning and measures of PFC protein synthesis to elucidate the possible underlying mechanisms.

One study that also examined the effects of simulated night work on the brain showed that SCN clock gene expression was unaffected (Salgado-Delgado et al., 2013). This is not surprising considering that the SCN is primarily synchronized to the environmental light/dark cycle, and light conditions remain unchanged in animal models of shift work utilizing forced activity (Salgado-Delgado et al., 2008; Granados-Fuentes and Herzog, 2013). However, changes in peripheral (hepatic) clock gene expression was reported, suggesting that simulated night shift work caused internal desynchrony, which may contribute to the negative metabolic effects often associated with night shift work (Salgado-Delgado et al., 2013; West and Bechtold, 2015; Marti et al., 2016). The present study indicates that internal desynchrony also occurs between brain regions, regulation of translation initiation in the PFC and hippocampus in this case. In light of previous and present results, simulated night shift work clearly has tissue-specific effects, which likely have effects on individual tissues, but may also affect entire networks more broadly through internal desynchrony. These effects largely remain to be elucidated.

The results of the present study also raise further questions. For example, are the observed changes in the mTOR/S6K1/BMAL1 pathway and changes in Arc interrelated or are these processes independent of one another? Additionally, the mTOR pathway has been implicated in regulation of both sleep and circadian rhythmicity. From a molecular biology standpoint the processes regulating sleep and wakefulness, and circadian timing, have for a long time been considered relatively separate. However, we are now beginning to see that many of the same pathways are implicated in both sleep and circadian processes. In the real world, sleep deficiency and

circadian dysregulation often co-occur, as seen for example in shift workers (for review see Kecklund and Axelsson, 2016). It is time to start examining the complex interactive effects of sleep and circadian rhythm disturbance and their effects on bodily regulatory processes.

Methodological Considerations

A few methodological aspects of this study deserve discussion. First, in the present study we used only two time-points of investigation, which precludes us from making any conclusions about the nature of circadian variation in cap-bound proteins. We used a 3-day work protocol, from this the questions emerge of whether the same effects would be observed after only 1 day of rest work; or consequently, if the protocol had been longer, would more detrimental effects or some form of adaptation occur. Second, some effects on translation may be compartmentalized to synaptic regions, which may go undetected in the present analysis of tissue lysate. Third, we have not investigated the potential mechanisms which may compensate for the negative effects of reduced cap-binding of certain initiation factors following rest work (Uniacke et al., 2012). In addition, one should consider that rats are nocturnal animals and may not respond to simulated shift work in the same way as a diurnal species would. However, our previous findings using this model suggest that effects observed in rats mimic effects described in humans (Marti et al., 2016; Grønli et al., 2017). Simulating shift work in ways which do not require forced activity (for review see Opperhuizen et al., 2015) may also provide information on the generalizability of the observed effects. Lastly, the experiment was carried out with two batches of rats, representing different strains. The ANCOVA suggested significant differences between experiments pertaining to translational repressors. However, our main findings on the translational promoters p-BMAL1 and p-S6K1 were similar across experiments/strains. The fact that the main results were upheld implies that these findings represent functionally meaningful impacts of night work.

CONCLUSION

Based on the current findings, we conclude that simulated night shift work in rats disrupts the pathways regulating the circadian component of the translation of mRNA in the PFC. These disruptions may explain the degraded waking function observed in night shift workers. Future animal studies should take care to consider diurnal variations when investigating the effects of simulated night shift work on cognitive performance and pathways regulating protein synthesis and synaptic plasticity, as timing-dependent factors may well play a role in these processes. Studies merging behavioral testing and assessment of neurochemical consequences of shift work in animal models will aid in furthering our understanding of the molecular basis of cognitive performance.

AUTHOR CONTRIBUTIONS

JG, ARM, PM, StP, JM, TTP and CRB designed the study. ARM, JM, TTP and SS collected the data. ARM and SuP performed the biochemical analyses. ARM, JG and CRB performed the statistical analyses. All authors contributed to the interpretation of data and writing of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncir.2017.00070/full#supplementary-material

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III

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Cognitive function and brain plasticity in a rat model of shift work: role of daily rhythms, sleep and glucocorticoids

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Many occupations require operations during the night-time when the internal circadian clock promotes sleep, in many cases resulting in impairments in cognitive performance and brain functioning. Here, we use a rat model to attempt to identify the biological mechanisms underlying such impaired performance. Rats were exposed to forced activity, either in their rest-phase (simulating night-shift work; rest work) or in their active-phase (simulating day-shift work; active work). Sleep, wakefulness and body temperature rhythm were monitored throughout. Following three work shifts, spatial memory performance was tested on the Morris Water Maze task. After 4 weeks washout, the work protocol was repeated, and blood and brain tissue collected. Simulated night-shift work impaired spatial memory and altered biochemical markers of cerebral cortical protein synthesis. Measures of daily rhythm strength were blunted, and sleep drive increased. Individual variation in the data suggested differences in shift work tolerance. Hierarchical regression analyses revealed that type of work, changes in daily rhythmicity and changes in sleep drive predict spatial memory performance and expression of brain protein synthesis regulators. Moreover, serum corticosterone levels predicted expression of brain protein synthesis regulators. These findings open new research avenues into the biological mechanisms that underlie individual variation in shift work tolerance.

We live in a 24-h society, where services are required around the clock. For this reason, a significant proportion of the work force is required to work shifts. The temporal aspects of the work schedule, night shift work in particular, has negative consequences for cognitive functioning. The risk of errors and accidents is increased on the night shift compared to morning and afternoon shifts, and the risk increases across consecutive night shifts worked[1,2].

It has long been assumed that the cause for cognitive dysfunction on the night shift is lack of sleep, and the link between perturbed sleep and cognitive dysfunction is well documented[3,4]. In the case of night shift work, both homeostatic and circadian aspects of sleep are challenged. Workers are required to stay awake for prolonged hours at times of day when the circadian system promotes rest, and to sleep at times when the circadian system promotes wakefulness[5,6].

In addition to disturbed sleep and circadian rhythm regulation, night shift work can act as a stressor on bodily systems, which may result in adverse health effects. Both acute sleep deprivation and chronic sleep restriction are known to induce neuroendocrine stress, as evidenced by elevated activity in the hypothalamic–pituitary–adrenal (HPA) axis, resulting in increased levels of circulating corticosteroids[7]. Night shift work has been shown to alter the daily rhythm of circulating corticosteroids in both laboratory and field studies[8–10].

Disturbances in circadian organization not only disrupt sleep and circulating corticosteroids, but also many other biological mechanisms, including brain de novo protein synthesis[11,12]. In order to maintain the

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Figure 1. Daily rhythm, sleep and wake parameters in active workers (AW) and rest workers (RW). (**A**) Body temperature rhythm amplitude during the 3-day work schedule relative to baseline. Body temperature rhythm was estimated with cosinor analysis. (**B**) Latency to rapid eye movement (REM) sleep after the 2nd work shift. (**C**) Mean length of non-REM (NREM) sleep bouts during the 3-day work period. (**D**,**H**) Cumulative slow wave energy (SWE, total integrated power in the 1–4 Hz frequency range) in quiet wakefulness and NREM sleep. (**F**,**J**,**L**) Cumulative wakefulness, NREM sleep and REM sleep, respectively. All cumulative data are expressed per 2 h during baseline and 3-day work schedule; workday 1 (W1) to W3 in RW (work hours: zeitgeber time, ZT2-10) and AW (work hours: ZT14-22). (**E**,**I**) Total SWE in quiet wakefulness and NREM sleep, respectively, in AW and RW during the 3-day work period. (**G**,**K**,**M**) Total time in wakefulness, NREM sleep and REM sleep, respectively, in AW and RW during the 3-day work period. Bar plots show mean ± SEM, with scatter plot overlaid. N = 9–17/group. ***$p < 0.001$, compared to baseline. $^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, between groups.

neurobiological substrates of cognitive functioning including synaptic plasticity, the brain is required to constantly synthesize new proteins. The rate of protein synthesis is commonly regulated at the level of initiation of translation[13,14]. This process depends on binding of eukaryotic initiation factor 4E (eIF4E) to the mRNA 5′-cap, and recruitment of several factors to form the translation initiation complex eIF4F[15]. Translation initiation is also in part promoted by the circadian clock protein brain-and-muscle arnt-like protein 1 (BMAL1)[16].

Utilizing a rat model of shift work, we recently showed that 3–4 consecutive simulated night shifts induced deficits in waking function, with modest effects on sleep time[17]. Moreover, we identified impairments to BMAL1-driven translational activity in the prefrontal cortex (PFC) of rats following three days of simulated night shift work[11].

While the negative effects of working night shifts are robust at the group level, there are considerable individual differences in tolerance to shift work. Shift work tolerance, the ability to adapt to shift work without adverse consequences, has been linked to a number of traits relating to age, gender, chronotype and personality[18]. Workers who self-report low shift work tolerance do not show objectively shorter or poorer sleep than those with high tolerance, but they do self-report less efficient sleep and show elevated levels of sleepiness and impaired cognitive functioning[19]. Identification of biological markers or predictors of shift work tolerance are lacking.

The biological mechanisms that underlie the negative effects of night shift work on cognitive performance and brain functioning remain unknown. In the present study we show, using a rat model, that markers of daily rhythmicity, sleep drive and consolidation of sleep, and circulating corticosteroids all predict different aspects of the negative impact of shift work on brain functioning and cognitive performance.

Results

Rest work impairs daily rhythmicity. During the 24-h baseline, body temperature rhythm amplitude did not significantly differ between groups (active worker, AW, mean 0.64 ± 0.11 °C versus rest worker, RW, mean 0.63 ± 0.13 °C; $t_{(28)} = 0.27$, $p = 0.79$, $d = 0.10$). The 3-day simulated night shift work (rest work) protocol impaired rhythm amplitude. Compared to baseline, rest workers exhibited a robust decrease in the rhythm amplitude by a mean of approximately 0.4 °C ($t_{(16)} = 7.88$, $p < 0.001$, $d = -2.82$; Fig. 1A), while the simulated day shift work (active work) protocol did not significantly alter the amplitude of body temperature rhythm, ($t_{(12)} = 2.06$, $p = 0.062$, $d = -0.73$; Fig. 1A).

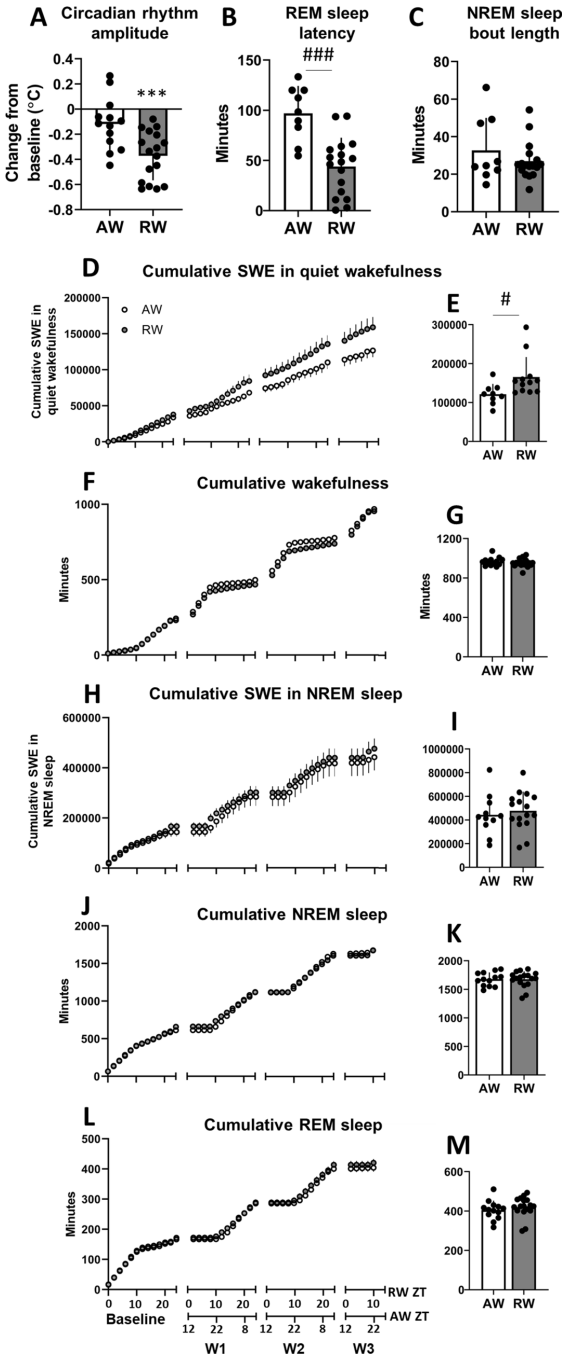
Since shift work can have differential effects on different bodily rhythms, rapid eye movement (REM) sleep latency was calculated as a sleep-related marker of daily rhythmicity[20]. Following the 2nd work shift, latency to enter REM sleep was significantly shorter following rest work than following active work ($t_{(24)} = 4.56$, $p < 0.001$, $d = 1.90$; Fig. 1B).

Importantly, individual differences in the rhythm parameters were considerable, with substantial overlap between the two work groups.

Rest work enhances sleep drive, without effects on sleep time or intensity. Across the 24 h baseline condition, all animals showed nocturnal patterns of wakefulness and diurnal patterns of sleep. Wakefulness occurred primarily during dark phase (72.9 ± 3.8% during zeitgeber time, ZT12-24) and sleep during light phase (71.6 ± 5.6% during ZT0-12). During baseline, there were no significant differences between RW and AW in time spent in wakefulness ($t_{(28)} = 0.49$, $p = 0.63$, $d = 0.18$), NREM sleep ($t_{(28)} = 0.63$, $p = 0.53$, $d = 0.24$) or REM sleep ($t_{(28)} = 0.44$, $p = 0.67$, $d = 0.16$). No significant differences were found in the length of NREM sleep bouts ($t_{(28)} = 1.26$, $p = 0.22$, $d = 0.46$) or REM sleep bouts ($t_{(28)} = 1.16$, $p = 0.26$, $d = 0.42$).

As previously demonstrated[17], forced activity for 8 h during the rest phase kept the rats awake except for sporadic micro-sleep episodes. Both groups spent more time awake and less time in sleep on work-days compared to baseline (AW wake $t_{(9)} = 5.8$, $p < 0.001$, $d = 2.48$, NREM $t_{(9)} = 4.05$, $p = 0.003$, $d = 1.80$, REM $t_{(9)} = 4.9$, $p < 0.001$, $d = 2.18$; RW wake $t_{(15)} = 10.13$, $p < 0.001$, $d = 3.55$, NREM $t_{(15)} = 8.21$, $p < 0.001$, $d = 2.76$, REM $t_{(15)} = 6.80$, $p < 0.001$, $d = 1.72$). Time spent in sleep during simulated shift work did not differ significantly between groups ($t_{(28)} = 1.40$, $p = 0.17$, $d = 0.38$). On average, RW spent 603 ± 53 and 610 ± 19 min (mean 606 ± 36 min), and AW spent 622 ± 41 and 624 ± 35 min (mean 623 ± 38 min) in sleep across the two successive working days. Rest work induced a redistribution of sleep to the endogenous active phase. During the undisturbed 16 h between the simulated work shifts, time in NREM sleep ($t_{(28)} = 1.49$, $p = 0.15$, $d = 0.54$) and REM sleep ($t_{(28)} = 1.28$, $p = 0.21$, $d = 0.47$) did not significantly differ between RW and AW. Following the 2nd work shift, latency to stable sleep (1 min of continuous sleep) was significantly shorter following rest work (13.8 ± 9.5 min) than following active work (32.1 ± 34.6 min) ($t_{(28)} = 2.09$, $p = 0.046$, $d = 0.72$).

Across the 3-day work period, groups did not significantly differ in the amount of cumulated wake time ($t_{(28)} = 0.71$, $p = 0.49$, $d = 0.26$; Fig. 1F,G), time spent in NREM sleep ($t_{(28)} = 0.09$, $p = 0.93$, $d = 0.03$; Fig. 1J,K) or


A
Circadian rhythm amplitude
Change from baseline (°C)
***
AW
RW
B
REM sleep latency
Minutes
###
C
NREM sleep bout length
D
Cumulative SWE in quiet wakefulness
Cumulative SWE in quiet wakefulness
AW
RW
E
#
F
Cumulative wakefulness
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G
H
Cumulative SWE in NREM sleep
Cumulative SWE in NREM sleep
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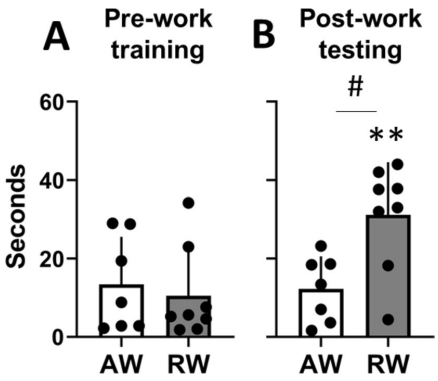

Figure 2. Latency to platform on the Morris Water Maze task, for active workers (AW) and rest workers (RW). (**A**) The last training trial before commencing the 3-day work period. Training occurred around zeitgeber time, ZT6. (**B**) The first testing trial immediately after the third work shift, AW at ZT20, RW at ZT10. Plots show mean ± SEM, with scatter plot overlaid. N = 7–8/group. **$p < 0.01$, compared to pre-work training. $^{\#}p < 0.05$, between groups.

REM sleep ($t_{(28)} = 1.50$, $p = 0.15$, $d = 0.55$, Fig. 1L,M), or accumulation of slow wave energy (1–4 Hz) during NREM sleep ($t_{(25)} = 0.52$, $p = 0.61$, $d = 0.20$, Fig. 1H,I). However, rest work led to higher accumulation of slow wave energy in quiet wakefulness, suggesting a degraded waking state and enhanced sleep drive ($t_{(19)} = 2.26$, $p = 0.04$, $d = 1.04$; Fig. 1D,E). These results replicate previous findings from our lab showing that work in the rest phase increases sleep drive, without affecting total time spent in sleep[17].

Previous mathematical modelling of sleep data (combining two-process model and stochastic model) suggests that duration of NREM sleep bouts importantly contributes to the observed effects of simulated shift work on sleep/wake dynamics in our rodent model[21]. Here, the average length of NREM sleep bouts did not significantly differ between groups ($t_{(24)} = 1.07$, $p = 0.30$, $d = 0.40$; Fig. 1C), although individual differences were considerable.

Rest work impairs spatial memory performance. At the end of the 3-day training period, before the 3-day work period, all animals were able to locate the hidden platform in the Morris Water Maze (MWM) task in less than 40 s, with no significant difference between groups ($t_{(13)} = 0.47$, $p = 0.65$, $d = 0.24$; Fig. 2A). When tested immediately after the third work shift, rest workers required longer time to find the hidden platform compared to their pre-work training session ($t_{(7)} = 5.04$, $p = 0.002$, $d = 1.64$), and compared to active workers ($t_{(13)} = 2.81$, $p = 0.014$, $d = -1.40$; Fig. 2B). Active workers' latency post-work did not significantly differ from that during pre-work training ($t_{(6)} = 0.21$, $p = 0.85$, $d = -0.11$). Latency to find the platform during pre-work training did not correlate with latency during post-work testing ($r = 0.18$, $p = 0.52$, all animals), indicating that post-work performance was independent of pre-work performance on the MWM task. Also, the differences in MWM performance cannot be explained by motor deficits following rest work as the swimming speed was higher in rest workers compared to active workers (mean 23.1 vs 16.0 cm/s; $t_{(13)} = 2.56$, $p = 0.02$, $d = 1.33$). Again, we observed considerable individual differences in spatial memory performance after simulated shift work (Fig. 2B).

Type of work, markers of daily rhythm dynamics and sleep drive predict spatial memory performance. Having observed group differences, as well as considerable individual variation in performance on the MWM task, we decided to test whether this variation might be related to specific aspects of work, sleep and daily rhythmicity during the 3-day work period. To achieve this, we conducted a multiple regression analysis with hierarchical regression approach. This method allows to test whether certain variables can predict part of the variation in a second variable (as indicated by the R^2 value), and whether adding more variables can enhance this prediction[22]. Adding variables to a model always enhances the R^2. Therefore, we report the adjusted R^2 which indicates predictive power after taking the addition of variables into account. If the adjusted R^2 is increased after adding a given variable, this indicates that the variable indeed improves prediction.

First, we tested a simple model (Step 1, Table 1) with work condition as the only predictor of latency to platform in the MWM task. Work condition alone modestly predicted MWM performance ($R^2 = 0.23$, Table 1). Next, we added circadian rhythm parameters (Step 2). Change in body temperature amplitude relative to baseline was chosen as a measure of global rhythm strength. REM sleep latency was chosen as a sleep-related marker of daily rhythmicity. This resulted in an increase in the adjusted R^2 by 0.08 (Table 1), suggesting that individual changes to circadian rhythm parameters slightly enhances the ability of the model to predict MWM performance.

Lastly, we tested whether adding sleep parameters further predicted MWM performance (Step 3). We applied cumulated slow-wave energy in quiet wakefulness and mean duration of the NREM sleep bouts across the last 24 h, since our previous findings suggest that these parameters are crucial markers of degraded waking function and sleep consolidation, and implicated in the observed effects of simulated shift work[17,21]. This resulted

	MWM latency to platform
Step 1, work condition	$F_{(1,11)} = 3.96$, $p = 0.07$
AW/RW	
R^2	0.23
Adj. R^2	0.20
Step 2, daily rhythm dynamics	$F_{(3,9)} = 2.52$, $p = 0.12$
Amplitude change	
REM sleep latency	
R^2	0.46
Adj. R^2 (change)	0.28 (0.08)
Step 3, sleep drive	$F_{(5,6)} = 3.74$, $p = 0.07$
Duration of NREM sleep bouts	
SWE in QW	
R^2	0.76
Adj. R^2 (change)	0.55 (0.27)

Table 1. Hierarchical regression analyses for predictors of Morris Water Maze (MWM) performance post simulated shift work. *AW* active work, *RW* rest work, *SWE* slow wave energy, *QW* quiet wakefulness, *REM* rapid eye movement sleep, *NREM* non-REM sleep.

in a drastic increase in the adjusted R^2 by 0.27, with the R^2 reaching 0.76 (Table 1), suggesting that these sleep markers strongly contribute to predict individual spatial memory performance, following simulated shift work.

While cumulated slow-wave energy in quiet wakefulness contributed to predict MWM task performance, it is possible that this could reflect differences in NREM sleep intensity. To test this, we ran a second model where Step 3 was changed to cumulated slow-wave energy in NREM sleep (Table S1). This reduced the adjusted R^2, suggesting that cumulated slow-wave energy in NREM sleep did not contribute to predict spatial performance in the MWM test in our animal model.

We also tested a third model where Step 3 was changed to cumulated time spent in NREM and REM sleep across the 3-day shift work protocol (Step 3, Table S2). Adding the amount of sleep spent across the shift work protocol resulted in a reduction in the adjusted R^2, suggesting that sleep time during the 3-day shift work period does not contribute to predict MWM task performance.

Rest work impairs BMAL1-driven protein translation in the prefrontal cortex. The expression of the translational promoters, cap-bound phosphorylated BMAL1 (p-BMAL1) and p-eIF4E, was not significantly changed in the prefrontal cortex (PFC) of the active work rats compared to their time-matched undisturbed controls (p-BMAL1 $t_{(12)} = 0.90$, $p = 0.39$, $d = 0.48$; p-eIF4E $t_{(10)} = 0.11$, $p = 0.92$, $d = 0.06$; Fig. 3A–C). In contrast, expression of p-BMAL1 was significantly reduced in the rest workers compared to their time-matched controls ($t_{(13)} = 3.19$, $p = 0.007$, $d = -1.68$; Fig. 3B), with large effect size for reduced p-eIF4E ($t_{(10)} = 2.56$, $p = 0.078$, $d = -1.48$; Fig. 3A). The reduced phosphorylation of BMAL1 indicates repression of translation in rest workers since p-BMAL1 is coupled to stimulation of protein translation[16].

Western blot analyses performed in the input samples (total tissue lysates used for cap-pulldown) showed no significant differences in protein expression, either from time-matched controls (indicating no time-of-day effect) (p-eIF4E AW $t_{(13)} = 0.59$, $p = 0.57$, $d = 0.30$; p-eIF4E RW $t_{(14)} = 0.80$, $p = 0.44$, $d = 0.40$; p-BMAL1 AW $t_{(12)} = 0.74$, $p = 0.47$, $d = 0.40$; p-BMAL1 RW $t_{(11)} = 1.22$, $p = 0.25$, $d = 0.68$; Fig. S1) or between the two work groups (indicating no effect of type of shift work) (p-eIF4E $t_{(13)} = 1.1$, $p = 0.29$, $d = 0.55$; p-BMAL1 $t_{(31)} = 0.29$, $p = 0.78$, $d = 0.15$; Fig. S1).

Levels of p-S6K1, the catalyst for BMAL1 phosphorylation, were significantly increased following active work ($t_{(11)} = 3.21$, $p = 0.008$, $d = 1.79$; Fig. 3D) compared to time-matched controls. Following rest work the p-S6K1 expression showed large effect size for reduced expression, although not statistically significantly different, compared to time-matched controls ($t_{(12)} = 1.97$, $p = 0.07$, $d = -1.10$ Fig. 3D).

The expression of the synaptic plasticity regulator activity-regulated cytoskeleton-associated protein (Arc) was not significantly changed following active work ($t_{(11)} = 0.27$, $p = 0.80$, $d = -0.15$; Fig. 3E,F), but it was significantly reduced following rest work ($t_{(12)} = 2.21$, $p = 0.047$, $d = -1.18$; Fig. 3E), compared to their respective time-matched controls.

Taken together, these results replicate our previous findings that simulated night shift work impairs regulation of cap-dependent initiation of translation as well as expression of the synaptic plasticity regulator Arc[11].

Simulated shift work alters serum corticosterone. Serum corticosterone showed normal time-of-day variation in time-matched controls, with higher levels at ZT12 before the animals enter their active phase, compared to ZT0 before the animals enter their resting phase ($t_{(14)} = 7.46$, $p < 0.001$, $d = 1.21$; Fig. 4). Following active work, at ZT0, serum corticosterone was significantly increased relative to time-matched undisturbed controls ($t_{(13)} = 2.61$, $p = 0.022$, $d = 1.31$; Fig. 4). In contrast, serum corticosterone was significantly reduced at ZT12 in animals that had been subjected to rest work, relative to time-matched controls ($t_{(15)} = 3.65$, $p = 0.002$,

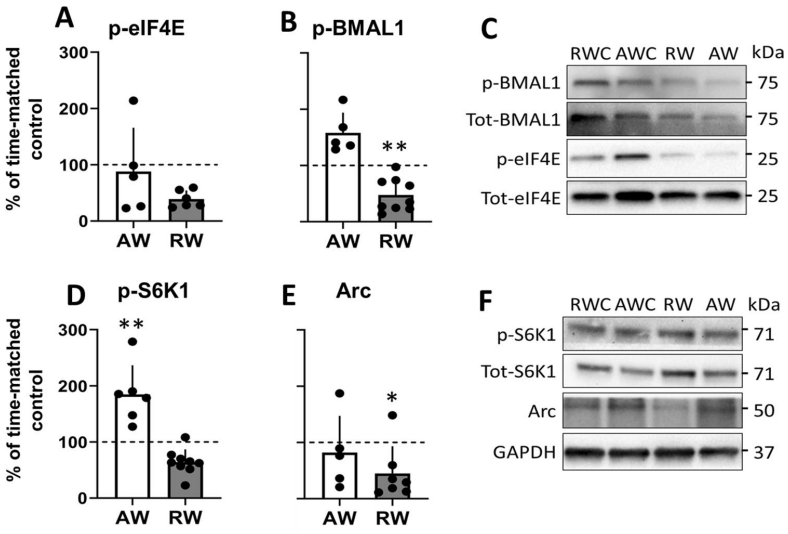

Figure 3. Expression of promoters of cap-dependent translation initiation, and synaptic plasticity regulators in prefrontal cortex following 3 days active work (AW, collected at zeitgeber time, ZT0) and rest work (RW, collected at ZT12). (**A**,**B**) m7GTP pull-down analysis. (**C**) Representative immunoblots for (**A**,**B**). Blots normalized to total eIF4E in the corresponding immunoblot. Phospho-protein normalized to total protein in the corresponding immunoblot. (**D**,**E**) Western blot analysis. (**F**) Representative immunoblots for (**D**,**E**). Full-length blots/gels are presented in Supplementary Figure S2. Blots normalized to GAPDH in the corresponding immunoblots. Phospho-protein normalized to total protein in the corresponding immunoblot. Quantification of immunoblots are expressed as percentage change relative to time-matched undisturbed control (AWC collected at ZT0, RWC collected at ZT12; normalized to 100%). Plots show mean ± SEM, with scatter plot overlaid. N = 6–9/group. *p < 0.05, **p < 0.01, compared to time-matched control.

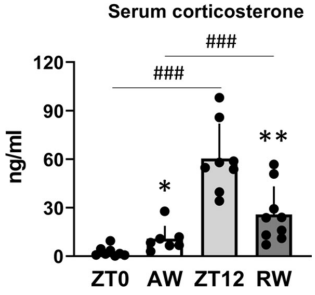

Figure 4. Concentration of serum corticosterone in undisturbed controls (zeitgeber time, ZT0 and ZT12) and following 3 days active work (AW, ZT0) and rest work (RW, ZT12). Plots show mean ± SEM, with scatter plot overlaid. N = 7–9/group. *p < 0.05, **p < 0.01, compared to time-matched controls. ###p < 0.001, between groups.

d = − 1.76), but was higher compared to AW ($t_{(14)} = 5.96$, p < 0.001, d = 3.14; Fig. 4). These results suggest that simulated shift work alters the concentration of serum corticosterone, likely through changes in total concentration, diurnal profile, or both.

Type of work, markers of daily rhythm dynamics and sleep drive and serum corticosterone predict different aspects of cap-dependent translation in the PFC. To evaluate whether work condition, parameters related to daily rhythmicity or sleep and level of stress hormone predict cortical expression of translational promoters and synaptic plasticity regulators, we conducted a multiple regression analysis with hierarchical regression approach, as described above.

	p-eIF4E	p-BMAL1	p-S6K1	Arc
Step 1, work condition	$F_{(1,7)} = 3.98$, $p = 0.09$	$F_{(1,11)} = 8.68$, $p = 0.013$	$F_{(1,11)} = 72.39$, $p < 0.001$	$F_{(1,8)} = 1.76$, $p = 0.22$
AW/RW				
R^2	0.36	0.44	0.87	0.18
Adj. R^2	0.27	0.39	0.86	0.08
Step 2, daily rhythm dynamics	$F_{(3,5)} = 2.59$, $p = 0.17$	$F_{(3,9)} = 5.07$, $p = 0.03$	$F_{(3,9)} = 22.58$, $p < 0.001$	$F_{(3,6)} = 0.49$, $p = 0.70$
Amplitude change				
REM sleep latency				
R^2	0.61	0.63	0.88	0.20
Adj. R^2 (change)	0.37 (0.10)	0.50 (0.11)	0.84 (−0.02)	−0.20 (−0.28)
Step 3, sleep drive	$F_{(5,2)} = 1.93$, $p = 0.38$	$F_{(5,6)} = 2.10$, $p = 0.20$	$F_{(5,6)} = 14.75$, $p = 0.003$	$F_{(5,3)} = 0.82$, $p = 0.61$
Duration of NREM sleep bouts				
SWE in QW				
R^2	0.83	0.64	0.92	0.58
Adj. R^2 (change)	0.40 (0.03)	0.33 (−0.17)	0.86 (0.02)	−0.12 (0.08)
Step 4, serum corticosterone	$F_{(6,1)} = 35.51$, $p = 0.13$	$F_{(6,5)} = 1.96$, $p = 0.24$	$F_{(6,5)} = 14.47$, $p = 0.005$	$F_{(6,2)} = 0.82$, $p = 0.64$
R^2	0.99	0.70	0.95	0.71
Adj. R^2 (change)	0.97 (0.57)	0.34 (0.01)	0.88 (0.02)	−0.16 (−0.04)

Table 2. Hierarchical regression analyses for predictors of prefrontal cortex protein expression following simulated shift work. *AW* active work, *RW* rest work, *SWE* slow wave energy, *QW* quiet wakefulness, *REM* rapid eye movement sleep, *NREM* non-REM sleep.

Work condition strongly predicted expression of p-S6K1 (the p-BMAL1 regulator) ($R^2 = 0.87$). All tested steps for p-S6K1 reached significance (p's < 0.005; Table 2), but only very slight changes to the adjusted R^2 across the steps show that this was due to the strong predictive effects of work condition on this protein. For all other examined proteins, work condition provided some predictive power (Step 1, Table 2).

Daily rhythm parameters predict cap-binding activity of translational promoters. Adding daily rhythm parameters to our hierarchical regression model (Step 2) enhanced the predictive power of expression of the cap-bound translational promoters p-eIF4E and p-BMAL1 (by 0.10 and 0.11, respectively; Table 2).

We performed a separate regression analysis to test whether these parameters also predict expression of total available protein in total tissue lysates. In the case of p-eIF4E, rhythm parameters also predicted expression in total tissue lysates (increased adj. R^2 by 0.16, Table S3), suggesting an overall impact of rhythms on p-eIF4E abundance. In the case of p-BMAL1, rhythm parameters did not predict expression in total tissue lysates (reduced adj. R^2 by 0.13, Table S3), suggesting that these specifically predict the cap-binding activity of p-BMAL1.

Sleep drive during the last 2 h marginally predicts the expression of translational promoters and the regulator p-S6K1. To examine the contribution of acute changes in sleep drive on translational promoters, we tested a model including these sleep parameters, as above (Step 3). Since protein expression and cap-binding activity are thought to reflect relatively short-term (minute- and hour-scale) biological processes, we used data starting after the third work shift when rats were returned to their home cage, until tissue collection 2 h later. Results show that markers of sleep drive during this period slightly predict the expression of cap-bound p-eIF4E (adj. R^2 increased by 0.03, Table 2), as well as p-S6K1 (adj. R^2 increased by 0.02, Table 2). These markers also predicted phosphorylation of eIF4E in total tissue lysates (adj. R^2 increased by 0.36, Table S3).

Since markers of sleep drive during wakefulness and sleep consolidation predicted protein expression, we tested a separate model to investigate whether this was also the case for accumulation of slow wave energy during NREM sleep (as a marker of sleep intensity). Slow-wave energy accumulation during NREM sleep across the 2 h time-window reduced the adjusted R^2 or resulted in negative adjusted R^2 across all proteins tested (Step 3, Table S4). Thus, slow-wave energy in NREM sleep did not contribute to predict any of the measured proteins.

Testing a separate step with markers of cumulated total sleep time (NREM sleep and REM sleep respectively) across the 3-day work period only slightly enhanced prediction of cap-bound p-BMAL1 (adj. R^2 increased by 0.01, Step 3, Table S5), with no enhanced prediction of any other measured proteins (Table S5).

Taken together these results suggest that the last 2-h measures of sleep drive to a slight extent, but not NREM sleep intensity or cumulated sleep across the work protocol, contribute to prediction of eIF4E and S6K1 phosphorylation in PFC after simulated shift work.

Serum corticosterone levels predict expression of the translational promoter p-eIF4E. To assess the contribution of serum corticosterone to the prediction of cortical protein activity, we added Step 4 (concentration of serum corticosterone) to our hierarchical regression model. Serum corticosterone drastically elevated the prediction of the translational promoter cap-bound p-eIF4E (adjusted R^2 increased by 0.57, Table 2). Serum corticosterone also enhanced the prediction of p-eIF4E expression in total tissue lysates (adjusted R^2 increased by 0.13,

Table S3), suggesting that concentration of corticosterone may predict abundance of p-eIF4E in the PFC following simulated shift work. Serum corticosterone only slightly enhanced the prediction of cap-bound p-BMAL1 (adjusted R^2 increased by 0.01, Table 2) and p-S6K1 (adjusted R^2 increased by 0.02, Table 2).

The expression of the synaptic plasticity regulator Arc was only predicted by work condition, not by any of the selected markers of circadian rhythmicity, sleep drive or serum corticosterone (negative adj. R^2 suggesting no fit of any of the models, Table 2).

Discussion

The aim of the present study was to identify potential mechanisms underlying the cognitive deficits associated with shift work. We utilized a rat model where animals were exposed to either simulated night shift work (rest work) or simulated day shift work (active work) for three consecutive days. Results show that individual variation in daily rhythm dynamics, short-term markers of sleep drive and serum corticosterone predict individual variations in spatial memory impairments and the cerebral cortical protein translational machinery after simulated shift work. These findings open new research avenues into the biological mechanisms underlying individual variation in shift work tolerance.

Effects of simulated shift work at the group level. At the group level, we observed effects of rest work on sleep and wakefulness as previously observed in our model. While simulated night shift work impacts on the temporal distribution of sleep throughout the 24-h day in our model, it does not affect overall time spent in sleep differently from simulated day shift work[17]. Moreover, slow-wave energy accumulation during NREM sleep did not differ between the groups. Nevertheless, rest work is associated with a degraded waking state, as evidenced by increased slow wave energy in quiet wakefulness during the simulated night shift period[17]. In the present study, we replicate this finding. This may be explained by global instability in arousal including OFF periods of neuronal firing typically associated with a local slow wave (which causes intermittent performance impairments)[23] or incomplete dissipation of homeostatic sleep drive[24] after simulated night shift work. Our previous data showed a lower build-up of sleep debt in the rest workers than in active workers, probably due to the slowing of EEG during quiet wakefulness[17]. A lower build-up of sleep drive in rest workers after work-hours may very well be explained by the time spent in sleep before work together with a recent finding in mice that quiet wakefulness is permissive to metabolic changes that occur in NREM sleep[25]. Human studies have reported relatively well-preserved deep sleep (stage N3 sleep) after the night shift[26]. Taken together these data suggest that NREM sleep quality and quantity per se do not impact on brain functioning after the night shift, but that other aspects of sleep regulation may play an important part. In the present study we also show that cognitive performance is impaired after rest work, as evidenced by reduced spatial memory performance on the Morris Water Maze task. This indicates that our model mimics the cognitive performance impairments associated with human night shift work[1,27]. We also show that simulated shift work impacts on concentrations of serum corticosterone, as evidenced by a reduction in corticosterone 2 h after rest work, also observed in several human studies[28–30].

Night shift work is associated with impairments to the circadian rhythm, in particular to what has been termed internal desynchronization[31,32]. In our study we find overall group differences in two separate bodily rhythms following rest work,the body temperature rhythm amplitude and REM sleep latency. Taken together, these results show that our model captures several aspects associated with human shift work.

Individual differences in tolerance to shift work. In most of the measured outcomes during and following simulated shift work, we observed considerable individual variation, with overlap between active and rest workers. Some of the measures in our observed group effects are undoubtedly influenced by the time-of-day in which animals return home from work and data is collected, as is also the case for humans that work shifts. Nevertheless, the individual differences indicate variation in shift work tolerance in our rat model, as has been documented in several human studies[18,19]. Changes to circadian rhythm, sleep and stress systems have been hypothesized as mechanisms contributing to the negative effects of shift work. Considering this, we tested whether changes to related parameters during the 3-day work protocol might predict the observed effects on cognitive performance and cortical protein expression in our model.

Markers of daily rhythm dynamics and sleep drive predict cognitive performance after simulated shift work. Markers of daily rhythmicity, and more strongly, markers of sleep drive in the last 24 h, predicted individual differences in spatial memory performance after simulated shift work. In human shift workers, sleep has to date been most clearly implicated as a predictor of cognition in general[3,4] and cognitive deficits in shift work specifically[5]. Our results suggest that changes in sleep drive during and between shifts are of importance, rather than sleep quality or cumulated time spent in sleep across the shift work protocol per se.

While sleep is important for maintaining cognitive performance[33], so is circadian rhythmicity[34]. The present model of night shift work has been shown to shift feeding rhythms toward the rest phase, with acute negative effects on markers of metabolic functioning[35]. Another recent study showed that similar manipulation of feeding rhythms also impaired cognitive performance and hippocampal functioning[36]. However, a different study failed to find any effects of time-restricted feeding on cognitive performance, despite multiple attempts[37]. Nevertheless, these findings highlight that circadian rhythm disturbance can reflect simultaneous impairments to multiple systems. Very few studies examine the interaction between circadian rhythms and sleep on cognitive performance, although some biochemical pathways implicated in memory performance and consolidation have been shown to be altered by both sleep and circadian rhythms. These for example include the cAMP/MAPK/CREB transcriptional pathway[38], but more recently also the cap-dependent translation initiation pathway[11,16,39,40].

Daily rhythm, sleep drive and serum corticosterone predict markers of cortical regulation of translation after simulated shift work. We have previously suggested that impairments to eIF4F complex assembly after rest work may in part underlie the cognitive deficits observed on the night shift[11]. The present study strengthens this notion. We replicate previous findings from our lab showing that the regulation of cap-bound translational regulators (eIF4E and BMAL1) is impaired in the prefrontal cortex following rest work, as compared to undisturbed time-matched controls[11].

We show that expression of the translational regulators p-BMAL1 and p-eIF4E within the PFC are predicted by changes in daily rhythmicity during simulated shift work. Phosphorylated BMAL1 was predicted by daily rhythmicity only in its mRNA-cap-bound form. While it was previously known that BMAL1 acts both as a clock protein and as a translational enhancer when phosphorylated[16], it was not clear whether its cap-binding activity is in fact affected by altered rhythms. The present results support the idea that p-BMAL1 translational activity is indeed regulated, or at least modulated, by overall changes to daily rhythmicity during simulated shift work.

Our data also point to a role for daily rhythmicity in the modulation of the translational regulator eIF4E. Phosphorylation state of eIF4E was predicted by parameters of daily rhythmicity, acute (2 h) markers of sleep drive and serum corticosterone. Few studies examine the interaction of circadian rhythms, sleep and/or corticosterone on eIF4E expression or on any related marker. However, eIF4E and several other proteins related to cap-dependent translation have been shown to oscillate in a circadian fashion in different brain regions[16,41,42]. A study by Tudor and colleagues showed that biochemical enhancement of cap-dependent translation by indirect promotion of eIF4E phosphorylation rescued sleep deprivation-associated cognitive deficits on the MWM task[40], providing a link between cap-dependent translation, sleep and cognitive performance.

We also observed individual changes in concentration of serum corticosterone after shift work, which predicted expression of several translational markers in the PFC, particularly eIF4E phosphorylation. While corticosterone is commonly referred to as a stress hormone, the observed changes in serum corticosterone cannot be said to reflect acute stress, considering that the levels were measured 2 h after ended simulated shift work. Two hours after the end of sleep restriction, or the onset of restraint stress, it has been shown that corticosterone levels are returned basal levels[43,44]. However, it is possible that changes may reflect longer-term alterations to the regulation of the HPA-axis-mediated stress system, as has been observed after human shift work[8–10] and in a rat model of long-term simulated shift work[45]. One study by Grønli and colleagues showed that p-eIF4E expression in the PFC was associated with prior sleep time and quality, but this association was abolished after chronic stress, highlighting a potential interaction between stress and sleep on translational regulation[39]. The PFC is known to be sensitive to stress in general and corticosteroids specifically[46], and stress and sleep interact to impact on synaptic plasticity and cognitive functioning in a complex way[47]. More studies are needed to further elucidate how circadian rhythm, sleep and stress systems together may affect cap-dependent translational processes specifically, and cognitive functioning more generally.

Our results showed that the BMAL1-regulator S6 Kinase 1 (S6K1) was strongly predicted by work condition, and slightly predicted by markers of sleepiness and sleep consolidation. A recent study showed that several protein kinases oscillate in abundance within forebrain synapses, mostly peaking at light–dark transitions[48]. Interestingly, sleep deprivation resulted in the loss of rhythmic expression of almost all previously identified rhythmic kinases, highlighting the sensitivity of kinases to sleep deprivation[48]. Another recent study showed that 10 h acute sleep deprivation impaired striatal S6K1 signalling, and performance on a motor task[49]. Inhibition of S6K1 also impaired motor performance, suggesting a link between S6K1 signalling, sleep and brain functioning[49]. We have previously shown that p-S6K1 expression varies significantly between time points of light–dark transitions (ZT0 vs ZT12) in the PFC of undisturbed animals, and that the high expression of p-S6K1 at ZT12 is abolished after rest work[11]. Considering previous literature and the present data, this outcome may be primarily related to effects of sleep deprivation on kinase stability.

Acting through the corticosteroid receptor, circulating corticosterone is likely to have a significant impact on S6K1 function as S6K1 phosphorylation is subject to inhibition by the corticosteroid receptor[50]. The apparent inversion of this relationship (low corticosterone coupled with reduced p- S6K1 in rest workers relative to active workers) is paradoxical. Yet the reductions in corticosterone concentration and p-S6K1 could both be explained as reactions to elevated glucocorticoid receptor stimulation, not at the time of euthanasia, but hours before, for instance early in the rest phase work session. A longitudinal profile of serum corticosterone from throughout the work sessions would be needed to address this possibility.

Arc protein is recognized as a critical regulator of activity-dependent synaptic plasticity in the mammalian brain[51]. The expression of Arc in individual animals was not predicted by any of the examined processes,circadian rhythmicity, sleep drive, total cumulated sleep time, NREM sleep intensity or serum corticosterone. Previous studies associate Arc protein expression with total sleep time[47]. In our case, both groups were exposed to 8 h forced activity, followed by 2 h home cage recovery prior to tissue collection. Thus, there may not have been sufficient individual variation in cumulated sleep time to identify differences in Arc expression between animals in our study.

Study limitations. Some limitations of the present study warrant discussion. Firstly, cognitive testing as well as assessment of protein synthesis and serum corticosterone levels were performed at one time point per group (rest workers at ZT10/ZT12, active workers at ZT22/ZT0). It cannot be excluded that part of the differences observed post-work can be attributed to time-of-day differences in time of testing. However, several studies show no time-of-day effects on performance on the MWM and related spatial tasks in nocturnal rodents[34,52–55]. Moreover, rest workers were trained and tested during their resting phase, at time-points closer to each other (ZT6 and ZT12 respectively), as opposed to active workers (ZT6 and ZT22 respectively). Thus, if recall were context-dependent one might expect RW to perform better on the task, while they in fact performed

much worse post-work both compared to their pre-work training sessions and to the active workers. When it comes to serum corticosterone, we cannot conclude whether changes are due to effects on total concentration or diurnal profile. REM sleep latency was selected as a sleep-dependent daily rhythm marker, and between-group differences were likely influenced by work at different circadian phases[20,56]. However, of importance to our study was the individual variation in cognitive performance, serum corticosterone concentrations and REM sleep latency, which overlapped between groups.

Secondly, hierarchical regression is inherently subjective, as predictors are chosen based on previous literature and hypothesized associations. However, the approach allows the inclusion of several predictors at multiple levels, and does not require large data sets, as with related machine-learning-based approaches. Our approach proved capable of identifying large effect sizes, indicating sufficient sensitivity even with a small number of animals. While the analysis can be criticised as underpowered, it raises several new hypotheses which can be tested with appropriate study designs. Future research could aim to manipulate markers of daily rhythm strength, sleep drive and/or serum corticosterone and measure the effect on MWM performance and PFC protein expression. For example, markers of sleep drive could be manipulated with environmental (e.g. light) or pharmacological agents. Such interventions would thus be hypothesized to directly impact on MWM performance and PFC protein expression, suggesting a causal link between these measures.

Thirdly, it would be of interest to inform whether the observed individual variability in shift work tolerance reflect trait or stochastic differences. However, the present experimental design does not allow us to assess this. One might consider comparing individual variation in shift work tolerance across days, but this could potentially reflect adaptations or additive effects of the simulated work protocol. Therefore, this issue should be addressed in future work with adapted study designs.

Lastly, we only included male rats in the present study. Human studies suggest that gender may modulate shift work tolerance, with women being more vulnerable to the short-term effects of shift work on sleep and cognitive performance, and men being more vulnerable to the longer-term health consequences[18]. While social and cultural aspects are likely important here, animal studies may aid in elucidating possible biological mechanisms underlying this potential dissociation.

Conclusion

In summary, we have demonstrated that in our rat model, simulated night shift work induces detrimental effects on cognitive performance and markers of brain plasticity, with considerable individual variation in shift work tolerance. Additionally, we show that markers of daily rhythmicity, sleep drive and serum corticosterone all predict various aspects of these outcomes. Notably, total sleep time did not predict any outcomes of cognitive performance and brain plasticity. Future studies should further investigate how these processes may interact to impair cognitive performance and brain functioning, both in shift workers and in other populations.

Methods

Ethics. This study was conducted in accordance with Norwegian laws and regulations, and The European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. The protocol was approved by the Norwegian Food Safety Authority (permit number: 11321).

Animals and housing. Male rats of the outbred Sprague-Dawley strain (n = 32, 16 experimental animals and 16 controls, nTac:SD; Taconic, Silkeborg, Denmark) were used in the study. At arrival the animals were weighing approximately 300 g. After acclimatization to the laboratory conditions, they were group housed in individually ventilated cages (IVC, Techniplast, 75 air changes/h) type IV (480 × 375 × 210 mm, 1,500 cm^2) and single housed during the experimental protocol (IVC cage type III, 425 × 266 × 185 mm, 800 cm^2). The animals were kept on a 12 h light/12 h dark schedule with lights on at 08:00 (zeitgeber time 0; ZT0). Mean light intensity during lights ON was 222 ± 112 lx. Food (Rat and mouse No. 1, Special Diets Services) and filtered water were available ad libitum throughout the experiment.

Surgery. Experimental animals were implanted with transmitters (Physiotel, Data Sciences International) for continuous wireless recording of body temperature and sleep/wake electroencephalography (EEG) and electromyography (EMG), as previously described[57]. In brief, animals were anaesthetized with subcutaneous injection of a mixture of fentanyl 0.277 mg/kg, fluanisone 8.8 mg/kg (Hypnorm, Janssen) and midazolam 2.5 mg/kg (Midazolam, Actavis), and the transmitters were placed in subcutaneous pockets in the dorsomedial lumbar region (4ET transmitters) or in the neck region (F40-EET transmitters). Intracranial electrodes for collection of EEG signals were secured to the skull with dental acrylic (GC Reline, America inc.); frontal-frontal derivation bregma coordinates: AP = 2.0 mm, ML = −2.0 mm and lambda coordinates: AP = 2.0 mm, ML = 2.0 mm; and frontal-parietal derivation bregma coordinates: AP = 2.0 mm, ML = −2.0 mm and lambda coordinates: AP = 2.0 mm, ML = 2.0 mm). Two electrodes were attached to the neck muscle for collection of EMG signals. Animals were allowed 14 days recovery before entering the experiment[58].

Overview of study design. The study was conducted in two parts; (1) to test the effect of simulated shift work on spatial memory performance; (2) to collect tissue for protein analysis following simulated shift work. Telemetric recordings were conducted throughout the experimental period.

In experiment 1, all animals underwent 5 days of baseline telemetric recordings, followed by 3 days of training on the Morris Water Maze (MWM) task. They were then exposed to 3 consecutive days of simulated shift work, during which they were randomized to either simulated night shift work (rest work; RW) or simulated day shift work (active work; AW). Immediately after the third shift, recall on the MWM task was tested.

Animals were allowed at least 4 weeks washout between experiments. In experiment 2, animals were again recorded for 5 days baseline and randomized to either rest work or active work for 3 consecutive days (yielding n = 16 data points per work condition for sleep and circadian parameters). Two hours after the third shift, animals were euthanized for blood and brain collection[11]. A separate batch of animals (n = 16; undisturbed controls), were single housed for 14 days before tissue collection.

Simulated shift work procedure. To simulate shift work, animals were exposed to forced activity for 8 h per day, centred either in their normal active phase (active work; ZT14–22) or in their normal rest phase (rest work; ZT2–10), as described previously[11,17,35]. Animals were placed in automatically rotating wheels (Rat Running Wheel, TSE Systems, Homburg, Germany,24 cm diameter; 3 rpm; 1,440 revolutions or approx. 1.1 km of linear distance per 8-h session). Food and water were available ad libitum. Rotating wheels, feeders and water bottles were cleaned after each work session with 5% ethanol solution. Between sessions, animals were housed in their home cage. Work schedules were repeated for 3 consecutive days.

Morris Water Maze task. For assessment of spatial memory performance, animals were subjected to the Morris Water Maze (MWM) task[59]. Training and testing occurred in a circular pool (diameter 130 cm, height 60 cm) filled with water (maintained at 25 ± 1 °C). Water was re-filled for each day of testing. On the walls inside and around the pool were spatial cues (coloured symbols) for navigation.

Prior to shift work, all animals received 3 days of training. Each animal had four trials per day starting around ZT6. All training and testing were performed by the same experimenter under dim red light conditions. Animals were released from one of eight starting positions and had to swim to reach a platform (11 cm^2, transparent plexiglass) on which they were allowed 15 s of rest before commencing the next trial. Trials were organized in semi-randomized order; the animals were never released from the same starting position twice in a row. On the first day, the platform was located in the middle of the pool, visible above the water surface. On the next two days, the platform was located in one specific quadrant of the pool, hidden below the water surface. Black paint (Tempera) was added to the water to make it opaque to prevent the rats from seeing the platform. If the animal failed to find the hidden platform within 120 s, it was guided to the platform by the experimenter, and a score of 120 s was given. One animal was unable to learn the platform location and consequently excluded from further analysis.

Immediately after the third work shift, the animals' recall of the platform location was tested (rest workers at ZT10, active workers at ZT22). EthoVision XT software (Noldus) was used for the acquisition and processing of data. Latency to platform and swim speed was calculated for each animal.

Telemetric recordings and processing of data. The wireless recording device (input voltage: − 1.25 to + 1.25 mV) acquired EEG and EMG signals at a sampling rate of 250 Hz. Body temperature was sampled at 50 Hz and data was stored per 10 s (Dataquest A.R.T, version 4.1, Data Sciences International). Telemetry signals were collected through receivers (RPC-2/RPC-3, Data Sciences International) placed directly beneath the animals' home cage or next to the rotating wheel during simulated shift work.

Processing and analysis of EEG and EMG data. Based on the EEG and EMG signals and in accordance with the criteria from Neckelmann and Ursin[60], wakefulness, non-REM (NREM) sleep and REM sleep was manually scored in 10 s epochs using Neuroscore software (version 3.2.0, Data Sciences International). For the purpose of scoring, EEG signals were filtered with high-pass at 0.5 Hz and low-pass at 35 Hz. EMG signals were filtered with high-pass at 5 Hz. We further distinguished quiet wakefulness from active wakefulness as those epochs of wakefulness in which the EMG peak-to-peak amplitude was ≤ 33rd percentile of all wakefulness EMG peak-to-peak amplitude values[25].

We calculated the average NREM sleep bout length, as previous mathematical modelling of data suggests that differences in NREM sleep bout duration importantly contribute to the observed effects of simulated shift work on sleep/wake dynamics in our model[21]. A NREM sleep bout was defined as 3 or more consecutive 10-s epochs of NREM sleep.

REM sleep latency was calculated as a sleep-related marker of circadian rhythmicity, as similar to humans REM sleep specifically has been shown to be regulated by the circadian system in rats[20]. This was defined as the latency to 3 consecutive 10-s epochs of REM sleep, upon return to the home cage after a work session[17]. Latency was calculated after the second work session since not all animals entered REM sleep after the third session (before tissue collection 2 h later). Similarly, latency to stable sleep was defined as latency to 6 consecutive 10-s epochs of sleep, upon return to the home cage after the second work session.

In addition to time spent in different vigilance states, we also analysed the spectral characteristics of the EEG during these states, using offline Fast Fourier Transform analysis on unfiltered EEG signals averaged across consecutive 10-s epochs. The procedure has been described in detail elsewhere[61]. Artefacts were removed by visual inspection of EEG signals, and an automated algorithm that detected epochs with EEG power spectral values that exceeded the mean value by at least 5 standard deviations. EEG spectral power tracked across states was normalized to the average power values of these vigilance states at the corresponding circadian time during baseline of each animal.

We calculated EEG slow-wave activity (SWA, average spectral power in the 1–4 Hz range) in the fronto-parietal EEG derivation in NREM sleep as a measure of sleep intensity, and in quiet wakefulness as a measure of the homeostatic sleep drive[61]. The accumulation of SWA over time (slow-wave energy, SWE) in quiet wakefulness and NREM sleep was calculated by multiplying the number of epochs in the specific state per 2 h with the average SWA in the given state for those hours.

Processing and analysis of body temperature data. Processing and analysis of body temperature data was performed using the *tidyverse* package in R software[62,63]. For each animal, body temperature was normalized to baseline (4-day baseline average set to 0). Artefacts were removed by a moving window over 100 data points at a time, excluding all values 2 °C above or below the mean, and 5 standard deviations above or below the mean. The 10-s temperature measurements were averaged to 5-min bins. The amplitude of the body temperature rhythm was applied as a measure of change in overall rhythm strength during work. This was analysed with cosinor analysis, using the *cosinor* package in R software[64]. Analysis was performed on 72 h baseline data and 72 h work data. Rhythm amplitude change from baseline was calculated by subtraction.

Tissue collection. After the third simulated work session, animals were returned to their home cage for 2 h before they were anesthetized with isoflurane, and euthanized by decapitation (AW at ZT0 and RW at ZT12). Undisturbed animals never exposed to work sessions were used as time-matched controls and sacrificed at ZT0 (n = 8) and ZT12 (n = 8).

Trunk blood was collected in serum tubes, left to coagulate on ice in serum tubes for 30 min, and subsequently centrifuged at room temperature (3,000 rpm for 10 min). Serum was aliquoted and stored at − 80 °C until analysis. The time from initial disturbance of the animal (by removing the cage from the rack) to complete blood collection was 2:15 ± 0:18 min.

Brains were quickly removed and bilateral PFC was dissected out since previous research from our lab showed biochemical alterations in PFC after shift work[11]. The tissue was flash frozen and stored at − 80 °C until analysis.

M7GTP (cap) pull-down and SDS-PAGE procedure. m7GTP (cap) pull-down assay was conducted in order to assess changes in the abundance and cap-associated total eIF4E, Ser209 phosphorylated eIF4E, total BMAL and Ser42 phosphorylated BMAL[65]. Bilateral PFC was homogenized in 1,000 µl of RIPA buffer (Pierce, Thermo Scientific) with added protease and phosphatase inhibitor (Halt, Thermo Scientific), and centrifuged for 10 min at 14,000g at 4 °C. For the cap pull-down assay, 400 µg of protein in addition to 30 µl of 7-methyl GTP-agarose beads (Jena bioscience #AC-141) were incubated for 4 h at 4 °C. Phosphate-buffered saline was used to wash the beads three times, then boiled in Laemmli sample buffer (Bio-Rad) and sample was resolved in SDS/PAGE gels (4–15% precast gels, Criterion TGX, Bio-Rad). Proteins were transferred to nitrocellulose membranes (Trans-Blot turbo transfer packs, Bio-Rad) which were then blocked with 5% non-fat dry milk, probed with antibodies and developed using chemiluminescence reagents (Pierce, #32106).

Antibodies used for immunoblotting were as follows: p-eIF4E (1:1,000, Cell Signaling #9741), eIF4E (1:1,000, Cell Signaling #9742), p-BMAL1 (1:1,000, Cell Signaling #13936), total BMAL1 (1:500; Santa Cruz Biotechnology #sc365645), p-pS6k (1:1,000, Santa Cruz Biotechnology #sc-7984), pS6k (1:1,000, Sigma #SAB4502691), Arc (1:500; Santa Cruz Biotechnology #sc17839), and GAPDH (1:5,000, Santa Cruz Biotechnology #sc32233).

Blots were scanned using Gel DOC XRS+ (Bio-Rad) and densitometric analyses were performed with ImageJ software (NIH). Densitometric values are expressed per unit of tot-eIF4E/GAPDH as specified, applied to the gel lane. Blots treated with phospho-specific antibody (p-eIF4E, p-BMAL1, p-pS6k) were stripped with stripping buffer (Restore western blot, Thermo Scientific) at 37 °C for 10 min, washed, blocked and re-probed with antibody recognizing total protein.

For this analysis the amount of cap-bound p-BMAL1 and total-BMAL1, was normalized to the total amount of cap-bound eIF4E. Phosphoproteins were normalized relative to the total protein on the same lane. Total proteins were normalized to loading control. Protein expression after simulated shift work was normalized to expression from time-matched controls.

Serum corticosterone analysis. Serum corticosterone was analysed in triplicate with the ELISA technique (Enzo ADI-900-097, Enzo Life Sciences) according to the manufacturers' instructions. The kit was selected based on previous data from our lab showing high correlation to other kits in addition to high detection of corticosterone even at low concentrations[66]. Values were averaged across the triplicates for each sample. The coefficient of variation (CV%) was accepted at < 20.

Statistical analyses. Statistical analyses were performed using R software (version 3.6.1)[63] and GraphPad Prism (version 8.2.1). Group differences in time spent in vigilance states was calculated using student's unpaired t-test. Within-group differences in vigilance states on work-days vs baseline was calculated using student's paired t-test. Group differences in latency to platform on the MWM task were computed using student's unpaired t-test. Correlation between pre- and post-work performance on the MWM task was calculated using Pearson's product moment correlation. Differences in serum corticosterone and PFC protein expression were compared between groups and relative to time-matched controls using Student's unpaired t-test. Difference in daily rhythm parameters following work relative to baseline was computed using Student's paired t-test. The alpha-value for significance was set to 0.05.

Cohen's d was calculated as measure of effect size. For interpreting d, 0.2 is considered small, 0.5 medium and 0.8 large effect size[67].

Multiple regression analysis using the hierarchical regression approach was performed to determine predictors of MWM task performance and PFC protein expression[22]. Included predictor variables were (1) work condition, (2) daily rhythm dynamics, including body temperature rhythm amplitude change and REM sleep latency, (3) sleep parameters, including (3a) average length of NREM sleep bouts and slow-wave energy in quiet wakefulness, (3b) total cumulated time spent in NREM sleep and REM sleep, or (3c) slow-wave energy in NREM sleep, and (4) serum corticosterone.

Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

A.M., T.P., P.M., C.B. and J.G. developed the study concept and designed the study; A.M., T.P., J.M., Ø.H. and J.G. collected the data; A.M., S.P., J.W., Ø.H. and J.G., analyzed the data; A.M. and J.G., drafted the manuscript. All authors interpreted the data, revised the manuscript and accepted the final version for submission.

Competing interests
The authors declare no competing interests.

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