Simulated Night Work, Sleep-Wake Changes and Markers of Brain Plasticity

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Abstract

Night work occurs in time windows when the body is primed for sleep and the opportunity to sleep when the body is primed for wake. This affects sleep and wakefulness patterns and cognitive functioning. I used an animal model to simulate night work and examine sleep and wakefulness changes. I additionally examined whether sleep and wakefulness could predict translational- (pS6K1, pBMAL1 and peIF4e) and plasticity markers (BDNF, Arc and NPAS4) implicated in cognitive performance.

3 days of night/day work was simulated by exposing male rats to forced activity in automatically rotating wheels for 8 hours during their rest phase (rest workers, $n = 9$) or active phase (active workers, $n = 6$). Sleep and wakefulness was telemetrically measured by electroencephalography and electromyography during the work period. Two hours after the last work day, the prefrontal cortex was dissected and analyzed for protein expression.

Relative to active workers, rest workers had a steeper incline in slow wave energy during wakefulness, suggesting a higher sleep drive. Between shifts, rest workers spent less time in non-rapid eye movement (NREM) sleep and restorative functions of NREM sleep were impaired. peIF4E and BDNF was predicted from NREM sleep episode lengths. BDNF was additionally predicted from slow wave activity during wakefulness and NPAS4 from number of waking episodes. Interestingly, no predictive power for BDNF was found at the group level.

Collected data replicates previous sleep, wakefulness and protein data from the shift work model, providing it reliability. The results further indicate that sleep and wakefulness parameters may predict cortical protein levels.

Keywords: night work, sleep, wakefulness, cognition, translation, brain plasticity

Abstract på norsk

Nattarbeid skjer i et tidsvindu hvor kroppen er innstilt på å sove og muligheten for å sove er når kroppen er innstilt på å være våken. Dette påvirker søvn og våkenhetsmønstre, samt kognitiv fingering. Jeg har brukt en dyremodell for å simulere nattarbeid og studere endringer i søvn og våkenhet. I tillegg har jeg studert om søvn og våkenhet kan predikere translasjons- (pS6K1, pBMAL1 og peIF4E) og plastisitets (BDNF, Arc og NPAS4) markører, implisert i kognitiv fungering.

3 dager med natt/dag arbeid ble simulert ved å utsette hannrotter for tvungen aktivitet i automatisk roterende hjul i 8 timer i løpet av deres hvilefase (hvilearbeidere, $n = 9$) eller aktive fase (aktive arbeidere, $n = 6$). Søvn og våkenhet ble telemtrisk målt via elektroencephalografi og elektromyografi i arbeidsperioden. To timer etter siste arbeidsdag, ble prefrontal korteks dissekert og analysert for proteinuttrykk.

Sammenlignet med aktive arbeidere, hadde hvilearbeidere en brattere økning i treige hjernebølger i løpet av våkenhet, som foreslår et høyere driv mot søvn. Mellom skiftene, tilbrakte hviearbeidere mindre tid i «non-rapid eyemovement» (NREM) søvn og de restorative funksjonene ved NREM søvn var svekket. peIF4E og BDNF var predikert fra lengden til NREM søvn episoder. BDNF var i tillegg predikert fra treige hjernebølger i løpet av våkenhet og NPAS4 fra antall våkenhetsepisoder. Interressant, var ingen prediktiv styrke funnet for BDNF på gruppenivå.

Innsamlet data replikerer tidligere søvn, våkenhet og proteindata fra skiftarbeidsmodellen, som styrker dens reliabilitet. Resultatene indikerer også at søvn og våkenhetsparametere kan predikere kortikale protein-nivåer.

Acknowledgements

I would first and foremost like to thank my main-supervisor Janne Grønli for letting me be part of Bergen Stress & Sleep group and their simulated shift work project. Janne is very passionate about her work and an inspiring person to work with. She has taught more than I may explicitly remember. She has taught me how to score rodent sleep, as well as quality ensuring all my sleep scores. In addition, she has been open to discuss theoretical aspects of the thesis, as well as given me feedback.

I also want to thank my co-supervisors Andrea R. Marti and Torhild T. Pedersen. Andrea has sampled all proteins from the brain tissue and given me many good suggestions throughout the writing process. She has also given be feedback on my writing. Torhild has introduced me to the sleep script and always been open to discuss questions I had about the thesis.

Lastly, I want to thank Jonathan Wisor for creating the Mat lab sleep report script, as well as setting aside his time to help me get the sleep script running properly. He has also equipped me with a lot of general knowledge about sleep from various perspectives, which has helped me a lot regarding the theoretical aspects of this thesis. Lastly, I want to thank Jelena Mrdalj for always finding time to answer my questions regarding statistics.

My contribution to the dataset in the thesis

I have been taking an active part in the ongoing simulated night shift project since august 2018, from the start of my student research scholarship (August 2018 – December 2018) to handing in my master's thesis (January 2019 – November 2019. I have been involved in planning the design of the experiment, preparation of telemetric transmitters, surgical implantation of the transmitters, setting-up equipment needed for recording the transmitters, post-operational care of animals after surgery, data collection from the night shift model and dissection of the animals. All preparation and statistics of sleep data (running it through the script, processing them in Excel and calculations in Statistica) has been done by me. Everything in this thesis also written by me.

My main responsibility during data collection and preparation has been related to sleep. Between (April and August 2019) I have manually scored and re-scored 552 960, 10 second epochs of sleep (24 h. = 8640 epochs. Each animal had 1x 24h baseline and 3x 24h work days; 4*8640 = 34560. 16 animals provided sleep data; 34560*16 = 552 960).

All protein data extraction from the PFC was done by my co-supervisor Andrea R. Marti, who is going to use the data for an article relate to her PhD thesis.

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1.1 Night Work and its Prevalence

Our society is dependent on services being available around the clock. As a consequence, work hours of several industries are organized so that workers succeed one another in the workplace, allowing the workplace to be operational 24/7 (PLoS Medicine Editors, 2011). Such an organization of work hours requires employees working shifts, which may occur partly or completely outside of regular work hours. Regular work hours are approximately between 06:00 - 18:00 (Statistics Norway, 2017) or 08:00 - 17:00 (Burch et al., 2009). This may change depending on regulations set within individual countries. Work shifts are further divided into morning-, evening or night shifts. Night shift, which is the focus of this thesis, fall completely outside of the regular work hours. According to the Norwegian Labor Inspection Authority (2019), night shift is considered as work hours occurring between 21:00 - 06:00, whereas in the EU it is approximately between 00:00 - 06:00 (Eurofond, 2017). It is estimated that 32-33% of the Norwegian workforce regularly work outside of ordinary work hours, with 14% working night shift (Statistics Norway, 2017). According to the European working conditions survey by Eurofond (2017), on average 21% of the workforce within the European Union reported working night shifts once a month or more, with Ireland (26%) reporting the highest prevalence and Italy (13%) reporting the lowest. Among the northernmost EU countries (Sweden, Denmark, Finland, Estonia), there is an especially high tendency of night work, ranging from 22 - 25%. The sectors with the highest amount of shift workers within the EU, is the health sector (40%), followed by transport (33%), industry (28%) and commerce and hospitality (27%).

There are several potential aspects of night work that may be addressed from a researcher's perspective. A commonly reported negative consequence of night work is sleep problems (Åkerstedt, 2003). Sleep problems further reduces cognitive abilities, which has been postulated to be an important variable in explaining a heightened risk of accidents and

injury during night work (Folkard & Tucker, 2003; Åkerstedt, 2019). In this thesis, my focus has firstly been to address how sleep and wakefulness is changed by working night shift. Secondly, I have addressed a poorly understood topic, namely how these sleep and wakefulness changes may be ascribed to changes in the neurobiology of cognitive functioning.

1.2 Sleep

Sleep is a global phenomenon across the whole animal kingdom (Anafi, Kayser & Raizen, 2018), where we enter a reversible state of reduced consciousness and responsiveness to stimuli from our surroundings (Carskadon & Dement, 2005; Von Economo, 1930). Decades ago, it was thought that sleep was simply an absence of wakefulness, where the brain entered a passive state with minimal or no brain activity. Even one of the discoverers of rapid eye movement (REM) sleep, Nathaniel Kleitman, believed this for a long time until his student, Eugene Aserinsky, in 1953 discovered recurrent patterns of brain waves and eye movements during sleep. Kleitman and Aserinsky's later discovery that REM sleep correlated with vivid dreams, further established a conception of the brain in fact being highly active during sleep (Aserinsky, 1996). Almost 7 decades later, it is now well recognized that sleep is organized into both non-rapid eye movement- (NREM) stages and REM sleep, that repeat themselves in naturally occurring sleep cycles during a night of sleep (Brown, Basheer, McKenna, Strecker & McCarley, 2012) across a wide range of mammalian species (Anafi et al., 2018).

It can be argued that sleep on the one hand serves an evolutionary disadvantage, as we become vulnerable to predators, do not sleep, drink or reproduce (Krueger, Frank, Wisor & Roy, 2016). However, since we still naturally find time to devote a large part of our 24 hour day to sleep, many theories of its importance have been presented. These theories have

postulated that sleep is important for immune function, reducing our caloric use, restoring our

brain energy balance following a period of wakefulness, restore synaptic plasticity and

cognitive performance (Krueger et al., 2016; Tononi & Cirelli, 2014). It has furthermore been

of interest to understand how components of sleep, such as the sleep stages serve distinct roles

regarding physiological and psychological functions.

1.3 Sleep Stages

Throughout a period of sleep, we cycle between the different sleep stages. NREM-

and REM sleep exhibit distinct patterns of brain, eye and muscle activity that can be measured

through polysomnography. In humans, polysomnography normally consists of extracranial

electroencephalography (EEG), electromyography (EMG) and electrooculography (EOG)

(Rechtschaffen & Kales, 1968). The same principle can be applied to rats, where intracranial

EEG and EMG recordings are used for interpreting sleep stages to analyze sleep quality and

architecture (Neckelmann & Ursin, 1993). EEG signals derive from ionic currents driving

action potentials of the cortex and indicate how neural firings occur over time. Signals are

recorded from an active electrode placed at a desired brain region and a reference electrode at

a different region. The shared recorded activity from the active- and reference electrode is

then removed, leaving relevant recorded activity from the active electrode. EEG is measured

in frequency (Hz) and amplitude (μV), with Hz indicating the number of neural firings per

second and μV the difference in peak-to-peak voltage (Krauss & Webber, 1999).

In both humans and rats, wakefulness is characterized by synchronized low amplitude

high frequency beta (15 - 30 Hz) and gamma (30 - 120 Hz) brain activity, in addition to a high

muscle tone (Berry et al., 2012). Based on intensity of muscle tonus occurring alongside these

EEG signals, wakefulness may in rats further be divided into the three sub-stages quiet,

intermediate and active wakefulness (Grønli et al., 2017). NREM sleep is usually divided into

3 sub-stages in humans, with each stage representing a gradual deepening of sleep. For each NREM sleep stage, the arousal threshold for waking up gradually increases and mental activity gradually decreases. In rodents, NREM sleep is usually considered as one single stage (Brown et al., 2012; Carskadon & Dement, 2005).

The American Academy of sleep Medicine defines the three sub stages of NREM sleep as N1, N2 and N3 (Berry et al., 2012). N1 is characterized by a slowing of EEG frequency and is considered a transitional stage, meaning that it is typically not detained for very long before sleep progresses into the next stage. In N2, characteristic sleep spindles and k-complexes start to occur on EEG signals. Sleep spindles are rapid bursts of low amplitude high frequency (7 - 14 Hz) activity and k-complexes are sudden peaks of negative activity, immediately followed by a positive peak (Brown et al., 2012; Loomis, Harvey & Hobart, 1937). N3 sleep is often considered as slow wave sleep (SWS) in the literature, with desynchronized high amplitude low frequency delta (0.5 - 4 Hz) waves (Brown et al., 2012; Carskadon & Dement, 2005). Delta waves will from now on be referred to as slow wave activity (SWA). Until 2007, there used to be 4 NREM sleep stages defined. A sleep stage consisting of NREM stage 3 and stage 4 was however merged to N3 (Iber, Ancoli-Israel, Chesson & Quan, 2007; Rechtschaffen & Kales, 1968).

Lastly, REM sleep, also termed paradoxical sleep by Michel Jouvet due to the sudden and paradoxical higher frequency EEG waves in sleep, exhibiting low amplitude high frequency activity resembling wakefulness. However, muscle tonus is inhibited, and the eye balls move rapidly in different directions during REM sleep, making it possible to distinguish REM sleep from wakefulness by an absence of muscle tone (Berry et al., 2012).

1.4 Human and Rat Sleep Architecture

A sleep period in humans is divided into sleep cycles consisting of NREM sleep, followed by a period of REM sleep lasting for approximately 90 minutes. As sleep progresses throughout the night, the quality of sleep within a sleep cycle changes. The first sleep cycles and the first part of the night, primarily consists of slow wave sleep (SWS). The last sleep cycles towards the end of the night, consist of light NREM sleep and longer REM sleep episodes. It is also common to have brief unconscious awakenings after one sleep cycle is ended (Carskadon & Dement, 2005).

The classification of rat sleep is usually more simplified than in humans. As in most of animal research laboratories NREM sleep is considered as one sleep stage, and referred to as NREM sleep (Brown et al., 2012) or SWS (Datta & Hobson, 2000; Neckelmann & Ursin, 1993). In this thesis, rat sleep will be referred to as NREM and REM sleep. Rats do not center their sleep within one period at night. They are polyphasic sleepers, meaning that they sleep and wake up several times throughout the 24 hour day. Their oscillations between sleep stages are therefore shorter and the sleep cycle typically last for 12-15 minutes. Rats may have both brief and long awakenings between the sleep cycles (Simasko & Mukherjee, 2009). Notably, rats are nocturnal animals, meaning that they spend more time awake during darkness and sleep more during the light hours. Hypnograms of a sleep period in humans (23:00 to 07:00) and rats (across 24h) is shown in figure 1 below.
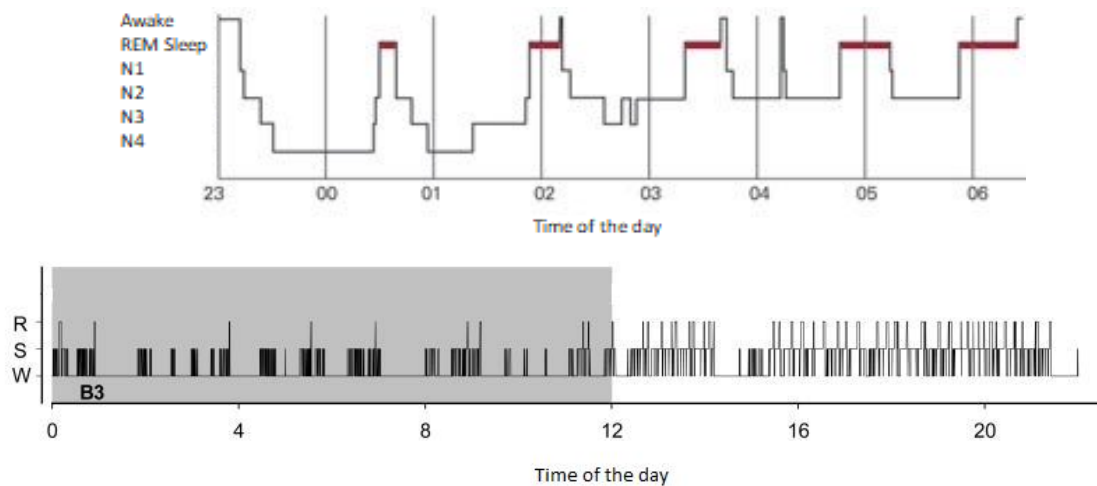
Figure 1. Hypnograms showing typical human (upper) and rodent (bottom) sleep. The shaded area in the rat hypnogram indicates 12 hours of darkness and white 12 hour of light. Upper figure modified and taken from Grønli & Ursin (2009) and bottom from Simasko & Mukherjee (2009).

1.5 Sleep Physiology

Sleep and wakefulness is comprised of complex neural circuitries in the brain, consisting of wake- and sleep promoting regions. The simplified description of sleep physiology given in this section, is only to introduce what underlies the EEG signals and to provide knowledge of the brains' neurotransmission that may regulate expression of the measured markers of brain plasticity.

Constantine von Economo was the first to discover and address wake- and sleep promoting regions of the brain in his studies of patients with encephalitis lethargica (Spanish flu). Symptoms associated with the disease, is either extreme sleepiness or insomnia. His post-mortem examinations of the brains from encephalitis lethargica patients, revealed that lesions in the junction between the brainstem and forebrain was found in those suffering from extreme sleepiness, whereas lesions in the anterior hypothalamic region was found in patients suffering from insomnia (Von Economo, 1930). Today, we know that the major sleep

promoting region of the brain is situated in the ventrolateral preoptic area (VLPO) of the anterior hypothalamus (Saper, Scammell & Lu, 2005) When sleep is initiated, VLPO projects GABAergic (gamma-aminobutyric acid) inhibiting signals towards wake-promoting regions of the brainstem which prevent their arousing effects. Von Economo therefore observed that his patients had severely reduced ability to inhibit these wake promoting regions, as the VLPO was lesioned. There are several wake-promoting neurotransmitter of the brain. Such as histamine, noradrenaline, serotonin, dopamine and orexin, all arising from their respective nuclei which are inhibited by the VLPO to initiate sleep. Once it is time to wake up, the basal forebrain and cerebral cortex is activated by the ventral arousal pathway. Within the basal forebrain, the main wake-promoting transmitters are acetylcholine (ACh) and glutamate (Saper et al., 2005; Scammell, Arrigoni & Lipton, 2017).

When entering NREM sleep, cessation of excitatory neuronal firings from wake-promoting regions contributes to the appearance of SWA on EEG signals (Esser, Hill & Tononi, 2007; Tononi & Cirelli, 2006. There are also GABAergic NREM sleep promoting neurons located in the parafacial zone of the brain stem, as well as in the basal forebrain where GABAergic neurons project directly towards cortex. GABAergic activity froms these neurons also contribute to SWA (Scammell et al., 2017).

During REM sleep, muscle atonia is initiated by glutamatergic firings from the brain stem. This innervates GABAergic neurons in the medulla and spinal cord, preventing muscular movement. The cortical high frequency desynchronized EEG signals observed in REM sleep, has been postulated to arise from cholinergic transmission deriving from the pedunculopontine (PPT) and laterodorsal tegmental nucleus (LDT) in the brainstem (Scammell et al., 2017). This has been proposed based on noradrenergic neurons attenuating and cholinergic neurons increasing their firings during REM sleep (Aston-Jones & Bloom, 1981; Kayama, Ohta & Jodo, 1992). More recently, Van Dort et al., (2015) optogenetically

stimulated either the PPT or LDT during NREM sleep. They found that stimulation initiated REM sleep but did not increase the length of REM sleep episodes. They therefore postulated that cholinergic activity may be involved in the initiation of REM sleep. Although both noradrenergic and cholinergic transmitters serve wake-promoting functions, they seem to have more distinct functionality in regard to REM sleep. It may be that the high frequency of the EEG signals during REM sleep to a larger represents cholinergic activity. This may influence expression of proteins involved in synaptic plasticity, which will be addressed later in section 1.9.5 regarding the activity regulated cytoskeleton association protein (Arc).

1.6 The Two Process Model of Sleep Regulation

One of the most recognized models of sleep, is the two-process model of sleep regulation. This model of sleep regulation (Borbèly, 1982) proposes that sleep quality and architecture is regulated by a sleep dependent homeostatic factor (S) and a sleep independent circadian factor (C). Process S reflects sleep pressure, or sleep need, being accumulated during wakefulness, which is later attenuated during NREM sleep. Specifically, the amount of SWA in NREM sleep is explained to attenuate sleep pressure. Such a mechanism by process S has been demonstrated in humans and rodents. In humans, 40 hours of sleep deprivation yields a significant increase in SWA during recovery sleep. The homeostatic sleep drive of process S is shown as an increase in sudden intrusions of low frequency EEG activity during time spent in wakefulness (Finelli, Baumann, Borbély & Achermann, 2000). Corresponding data has also been demonstrated in rats, where 3h and 6h sleep deprivation during the resting phase (Tobler & Borbély, 1990) and 24 h total sleep deprivation (Borbély & Neuhaus, 1979) increases the amount of SWA in the subsequent recovery sleep, compared to baseline sleep. On the other hand, taking naps in the afternoon decreases the amount of SWA during the sleep period. In fact, it has been found that the sum of SWA during afternoon napping and

night sleep is equal to that of baseline night sleep (Feinberg et al., 1985). Similarly, it has also been demonstrated that sleep depriving individuals to a fixed percentage of their baseline SWA, shows that the sum of additional SWA during subsequent sleep is equal to the 100% of baseline SWA (Andrew, Frank & Sharon, 1987). Based on this reasoning, it is therefore postulated that sleep is homeostatically regulated, where SWA in NREM sleep serves an attenuating effect and time in wakefulness an augmenting effect on sleep propensity.

Sleep drive is however not a linear process, due to its interaction with process C. Circadian rhythms repeat themselves after approximately 24 hours and maintains it rhythmicity regardless of process S. Both contribute to sleepiness. Figure 2 below represents the relationship between process S and C. T_1 - T_2 represent sleep initiation, causing to S decrease until it reaches its intersection with C and awakening occurs. The distance between S and C, indicate the sleep pressure; more distance indicates higher sleep pressure. T_1 represents a regular time to go to bed for many of us. T_2 represents a scenario where the individual does not go to bed before the early morning hours, such as in the case of a night shift worker. The breakdown of S is therefore less efficient, as C is on an upward going path in the morning. This is evident in the model, as the intersection with C happens after shorter sleep time, compared to T_1 when sleep was initiated in the evening. When process C naturally increases during the day, it is referred to as the wake maintenance zone. It then becomes more difficult to fall asleep due to the circadian upwards going oscillation (De Zeeuw et al., 2018). For night workers, the hours when process C increases falls often together with their sleep time after the shift has ended. Consequently, this results in shorter sleep duration compared to sleep occurring at night. This will be addressed more in detail in section 1.7.1 below.
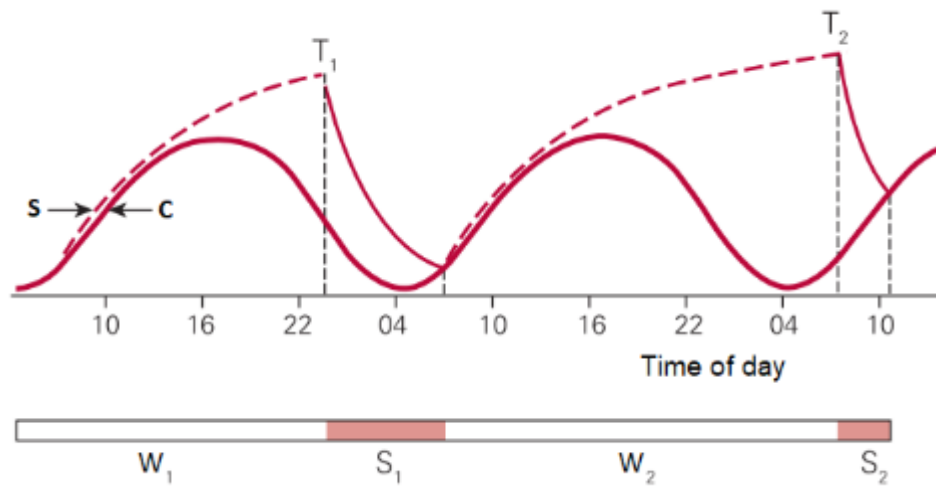
Figure. 2. Two process model of sleep regulation (Modified and taken from Grønli & Ursin, 2009).

1.7 Sleep among Human Shift Workers

The effect of the wake-maintenance zone has been indicated in several human shift work studies. Surveys on shift workers have found overall subjective reports of fatigue and insomnia, but not necessarily sleepiness among night workers (Øyane, Pallesen, Moen, Åkerstedt & Bjorvatn, 2013; Shen et al., 2006). This is interesting, as sleeping often reduces fatigue, but night workers do however seem less able to do so after shifts. In coherence with such a statement, there are also several reports of night workers not getting sufficient amounts of sleep when getting off work (Geiger-Brown et al., 2012; Ohayon et al., 2002; Åkerstedt, Nordin, Alfredsson, Westerholm, & Kecklund, 2010).

Polysomnographic recordings of night workers coincide with such reports, where total sleep during daytime in night workers is significantly reduced (Foret & Benoit, 1974; Matsumoto, 1978; Mitler, Miller, Lipsitz, Walsh & Wylie, 1997; Tilley, Wilkinson, Warren, Watson & Drud, 1982; Torsvall, Akerstedt, Gillander & Knutsson, 1989; Åkerstedt, Kecklund & Knutsson, 1991). In regards to changes in sleep architecture between daytime and night-time sleep in shift workers, several studies have also reported that daytime sleep following

night shifts do not cause a significant change in total time spent in SWS (Matsumoto, 1978; Foret & Benoit, 1974; Torsvall et al., 1989) or just marginally (Åkerstedt et al,. 1991). In combinations with the above mentioned finding of insomnia and trouble initiating sleep, it therefore seems that once sleep is initiated during daytime after a night shift, it is of shorter duration and does not adapt to compensate for any increased sleep pressure by increasing subsequent time in SWS. Furthermore, it has been demonstrated that SWA during NREM sleep is not significantly increased during daytime sleep after prolonged wakefulness (Parrino et al., 1993; Åkerstedt et al., 1991). This is different from what is seen in regular studies of sleep deprivation (Finelli et al., 2000; Tobler & Borbély, 1990; Borbèly & Neuhaus, 1979), where sleep adapts for an increased sleep pressure by increasing SWA. In sum, it therefore seems that day-time sleep after night work does not compensate for having been awake during night time, as both length and quality of NREM sleep remains largely unchanged.

1.8 Animal Shift Work Studies

To allow for a deeper understanding of changes in the biological mechanisms after night work, researchers have developed various rodent models of shift work. One model keep rodents in regular 12 hour of light and darkness, but manipulate the daily patterns of activity and inactivity within these 24 hours. Usually, activity is manipulated by forced activity during the light phase (inactive phase, simulating night work) or during the dark phase (active phase, simulating day work). The vast majority of such studies have however largely focused on metabolic consequences of shift work (Opperhuizen, van Kerkhof, Proper, Rodenburg & Kalsbeek, 2015). Simulated night work in rodents have shown to cause internal desynchrony of the endogenous circadian rhythm. This has been manifested in altered patterns of food intake and glucose rhythmicity (Salgado-Delgado, Angeles-Castellanos, Buijs, Escobar,

2006), circadian genes in the liver (Barclay et al., 2012) and circadian driven plasticity in the brain (Marti et al., 2017).

One study by Grønli et al. (2017) has examined changes in sleep using a forced activity model of shift work in rats. By utilizing EEG and EMG measurements continuously across 4 consecutive shifts, their data showed that forced activity during the resting phase in comparison to in the active phase, caused a cumulated increase in SWA during wakefulness. This was specifically prominent during the quiet wakefulness (QW, explained in section 2.7) indicating that rest workers build up more sleep pressure than active workers. Between the enforced activity sessions, it was also observed that rest working rats spent more time in wakefulness overall. Higher gamma frequencies (80 – 90 Hz) were also significantly increased, reflecting an increased cortical waking state in the hours between simulated night shifts. Importantly, their time spent in, and the duration of NREM sleep episodes were significantly lower for a large portion of the time between rest work compared to active work. Moreover, SWA in NREM sleep was lower after rest work, reflecting reduced quality of sleep. In coherence with human shift studies in the previous section, these data also indicate that 1) rest work in rats causes an increase in sleep propensity, and 2) the hours between shifts are comprised of more wakefulness and less sleep. Findings of reduced SWA activity in the shift work model, may also indicate that sleep quality is reduced during sleep after night work.

The differences between rodent sleep and human sleep in regard to sleep architecture are significant (as addressed in section 1.4). One could therefore argue that rodent sleep does not translate to human sleep. However, both human and rat sleep depend largely on the same underlying homeostatic, circadian and neurochemical processes (Revel, Gottowik, Gatti, Wettstein & Moreau, 2009). In fact, the two-process model of sleep regulation was initially proposed based on rodent data (Daan, Beersma & Borbély, 1984). Animal models have

therefore been proven to be valid for elucidating on several types of human sleep disorders

(Toth & Bhargava, 2013).

1.9 Accidents, Errors and Injury at Night-Time as a Consequence of Sleep

Too little sleep and sleepiness during wakefulness have been proposed to predict fatal

accidents and errors at night shifts. A longitudinal study involving almost 48 000 Swedish

citizens, found that reports of having difficulties sleeping and not working during daytime

were predictors of a total of 160 fatal traffic accidents that occurred among the participants

(Åkerstedt, Fredlund, Gillberg & Jansson, 2002). Further, a recent review shows an increased

likelihood of falling asleep behind the wheel while commuting home from night shifts and

during night shifts (Åkerstedt, 2019). Such observations may be explained from circadian

factors, as vigilance and cognitive abilities vary in coherence with circadian oscillations

(Blatter & Cajochen, 2007; Åkerstedt, 2019). There is also reason to believe that sleep

pressure alone can increase the risk of accidents. This has been demonstrated in a study where

taking naps prior to night shifts, reduced the likelihood of being involved in traffic accidents

(Garbarino et al., 2004). Furthermore, an in-depth review of shift work studies by Folkard &

Tucker (2003) concluded that workers had reduced productivity and were more prone to

making mistakes during night shifts. Importantly, this risk is found to increase for each

successive night shift. In coherence with a steadily increase in sleep pressures during a night

shift period, this may indicate that sleep serves an important function in maintaining work

performance and safety. Although the ability to initiate and maintain sleep for a longer time

may overall be driven by circadian factors, findings that accident risk is increased across night

shifts may be a result of continuously increasing sleep debt. To further understand the

heightened risks of night-time accidents, it is of interest to address how underlying cognitive

mechanisms are altered and how cognitive mechanisms can be seen in relation with sleep

specific variables.

1.10 Night Work and Cognitive abilities

A widely used cognitive test among sleep deprivation and shift work studies, is the psychomotor vigilance task (PVT). PVT is a simple and monotonous task where participants are asked to press a button once prompted on a screen. It measures reaction times and lapses which is used to indicate vigilance and the ability of the participant to sustain attention (Ruggiero & Redeker, 2014). PVT performances measured among night workers in the health care (Geiger-Brown et al., 2012; Behrens et al., 2019) and transport sector (Dorrian, Roach, Fletcher & Dawson, 2007) have indeed shown that performance is reduced when measured during night shifts. Interestingly, reduced PVT performance could be seen in coherence with some of the relatively simple tasks that could have potentially fatal outcomes in these jobs. Such as healthcare personnel assuming wrong patient identity and medication errors (Johnson, Jung, Brown, Weaver & Richards, 2014) or truck drivers falling asleep behind the steering wheel (Finkelman, 1994), both having been associated with not getting into enough sleep. A PVT adapted for rodents, where whisker stimulation is used as a conditioned stimulus for a licking a sucrose solution, show that 6 to12 hours of sleep deprivation significantly reduced performance, displaying a dynamic with increased performance in the circadian wake maintenance zone. Importantly, lapses were shown to be associated with increased SWA during wakefulness (Walker, Walker, Fuentes & Rector, 2011). Sleep drive during night work may therefore contribute to a reduced ability to maintain focus on tasks.

Work-related tasks may range from simple to highly complex. Hence, simple PVT performance and sustained attention might not be representative of the majority of tasks the worker is set to do at a night shift. The tasks could involve divided attention, selective attention or task shifting etc. (Schmidt, Collette, Cajochen, & Peigneux, 2007). To increase

naturalistic validity of work performance and test a wider range of cognitive processes, researchers have simulated work specific scenarios within different work sectors. In a simulated train setting, Dorrian et al. (2007) tested train drivers who had worked 2 night shifts prior. Train drivers who were fatigued, less efficiently operated the machinery, in addition to failures of acting on incoming speed regulations leading to more broken speed limits. In a driving simulator where it was simulated night workers commuting home after a shift, it was found that wheels were significantly more outside of lane markings, eye blink durations were increased and time to accidents were reduced relative to a day-time baseline (Åkerstedt, Peters, Anund & Kecklund, 2005). Rodent studies alike show that sleep and circadian manipulation reduce cognitive performance in more complex behavioral tests. Zero to 6 hours of sleep deprivation has shown to reduce performance in the novel object recognition task (Palchykova et al., 2006). Shortening the day (24h vs. 20 hour day) has also demonstrated reduced cognitive flexibility in the morris water maze in mice (Karatsoreos, Bhagat, Bloss, Morrison, McEwen, 2011). A recent study we performed using the simulated night shift model in rats, also cohere with these data. Our results showed that performance in the morris water maze spatial task was reduced in rest workers compared to active workers after three shifts (Marti et al., unpublished data).

Summed up, sleep is important to maintain cognitive abilities. Moreover, cognitive abilities are compromised during night shifts, an important variable to understand to preserve work safety. To further understand how these cognitive abilities are compromised, this thesis will attempt to elucidate this by examining biological markers of brain plasticity. Importantly, how brain plasticity may be associated with sleep specific parameters in a shift work model. This will be addressed in the section below.

1.11 Protein Synthesis

Protein synthesis is one of the most fundamental biological processes in living organisms. It entails the process of cells synthesizing proteins, based on genetic sequences in the DNA. Protein synthesis is crucial for brain plasticity, which is the ability of neurons in the brain to change their connections based on experience. Plasticity is important for establishing memories and cognitive functioning (Sutton & Schuman, 2006; Nikolaienko, Patil, Eriksen & Bramham, 2018).

Protein synthesis includes several steps, broadly it can be divided into transcription and translation steps, both being tightly connected but independently occurring processes. In short, transcription is the process of copying one strand of a specific genetic sequence in our DNA to a messenger RNA (mRNA) within the nucleus of a cell. Subsequently in translation, mRNA bases are translated into amino acids in ribosomes, ending with a protein being formed. Both processes are divided into three phases: initiation, elongation and termination. In the section below, transcription and translation will be addressed shortly with and approach that is relevant for the biological measurements of this thesis.

1.11.1 Transcription. Transcription is initiated by the enzyme, RNA polymerase, binding to a promoter region of a genetic sequence in the DNA. It uncoils the DNA double helix and exposes its nucleotide bases. RNA polymerase then copies one strand of nucleotide bases in the genetic sequence to an mRNA strand, in the process of elongation. Elongation ends when RNA polymerase reaches the terminator region of the gene and a mature mRNA has been formed (Purves et al., 2001; Whishaw & Kolb, 2010).

1.11.2 Translation. After a mature mRNA has been formed, it leaves the cell nucleus and enters the cytoplasm where translation occurs. In eukaryotes, a guanine nucleotide is connected to the mRNA 5' end via a 5' to 5' triphosphate bond, referred to as the 7-methylguanylate *cap* (m^7GTP). In order for translation-initiation to occur, it is dependent on

eukaryotic initiation factors (eIF) binding to this cap of the mRNA. The eukaryotic initiation factor eIF4E binds first to the mRNA cap and then recruits other initiation factors, eIF4G and eIF4A, to form a cap-bound complex (eIF4F) (Cooper, 2000; Pain, 1996, Pestova et al., 2001) (see figure 3C below).

Subsequently, a ribosome binds to the cap-bound complex and migrates along the mRNA. Here codons in the mRNA specify which amino acids to be gathered and transferred into the ribosome. When the ribosomal translation complex reaches the stop codon, amino acids specified by the mRNA codons have then been bound together in their respective sequence and a protein is formed (Pain, 1996). The most important point in this paragraph is that eIF4E is an important for forming a cap-bound translational complex one the mRNA cap, and that it has an essential role in expressing a protein at the translational step.

Translation-initiation is affected by signaling from the mammalian target of rapamycin complex (mTORC1). It does so by regulating one of it down-stream targets, a component called ribosomal protein S6 kinase 1 (S6K1). S6K1 functions by promoting ribosomal subunits, a smaller unit of a complete ribosome, which is crucial for reading and copying mRNA strands in translation. In addition, S6K1 signaling facilitates the process of forming the cap-bound complex (Hoeffer & Klann, 2010).

1.11.3 Sleep deprivation and translation-initiation. Specifically eIF4E has been demonstrated to be affected by sleep deprivation. In mice, Tudor et al. (2016) sleep deprived mice for 5 hours and found that mTORC1 activity was attenuated and associations between eIF4E and eIF4G were reduced in the hippocampus. By virally expressing eIF4E prior to sleep deprivation, it was on the contrary shown that eIF4E levels were unaffected. Moreover, such mice showed the same cognitive performance as non-sleep deprived control mice in the object recognition task, in comparison to sleep deprived control mice. Grønli, Dagestad, Milde, Murison & Bramham (2012) have similarly shown that eIF4E is reduced in rats

following sleep deprivation by gentle handling in the dentate gyrus. For the PFC, however,

they found no differences in eIF4E levels, indicating that brain regions react differently to

sleep deprivation (Grønli et al., 2014). Following simulated shift work however, its

expression has not been shown to be significantly altered (Marti et al, 2017).

       1.11.4 Circadian Dependent Translation-initiation. There are indications of

circadian factors acting independently of sleep on translation-initiation. The brain and muscle

arnt-like-protein-1 (BMAL1), is one of the key proteins involved in maintaining our circadian

rhythm and is naturally upregulated and downregulated throughout the 24 hour day

(Takahashi, 2017). Recently, BMAL1 was found to promote translation-initiation, indicating

that protein synthesis is partly driven by the circadian rhythm (Lipton et al., 2015). This cap-

dependent translation-initiation is regulated by the multiprotein complex mTORC1 signaling

pathway, where it phosphorylates the S6K1 protein (LaPlante & Sabatini, 2009). In its

phosphorylated state, S6K1 interacts with BMAL1 by phosphorylating it, which then leads to

promotion of protein synthesis by binding to the eIF4F complex (Lipton et al., 2015, see

figure 3a - c below).
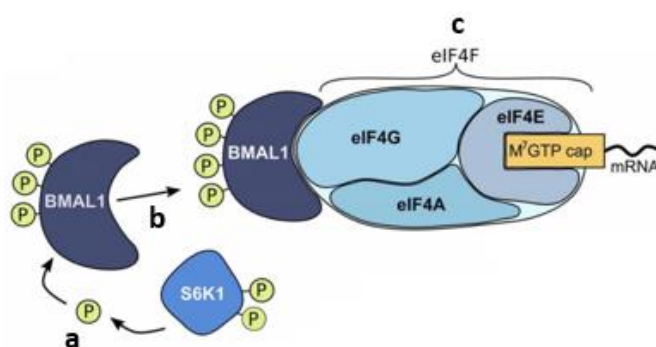
Figure 3. Circadian Regulation of translation-Initiation (taken from Marti et al., 2017).

(A) pS6K1, target of the mTORC1 complex, phosphorylates the circadian protein

BMAL1. (B) Phosphorylated BMAL1 binds to (C) the cap-bound complex (eIF4F),

which is bound to the 7-methylguanylate cap (m^7G) on the mRNA.

This mechanism of circadian driven translation has been shown to be altered in the simulated night shift model. Rest working rats show significantly reduced levels of pS6K1 and cap-bound pBMAL1, compared to active workers. Interestingly, these changes were specific to the PFC and not the hippocampus (Marti et al., 2017). As PFC is implicated in many cognitive functions, such as executive functioning and behavioral flexibility (Kesner & Churchwell, 2011), it is therefore interesting whether these findings may explain compromised work safety during night time. Reduced cognitive functioning has further been shown in S6K1 deficient mice, where they have impaired contextual fear memory, aversive conditioned taste memory and spatial learning (Antion et al., 2008).

The direct role of sleep in regulating S6K1 is however more uncertain. S6K1 is not always seen in sleep deprivation studies (Tudor et al., 2016). Other studies have however more recently found pS6K1 to be reduced in the mice hippocampi after 5 hours of sleep deprivation (Frolinger et al., 2018) and in the cerebellum and striatum following 10 hours of sleep deprivation (Kam et al., 2019). Why there are conflicting findings for S6K1, has been briefly discussed to depend on the method and length of sleep deprivation (Kam et al., 2019). It should further not be excluded that addressing specific sleep parameters may contribute to elucidate the involvement of sleep on S6K1 expression, alongside circadian driven translation-initiation.

1.12 Synaptic plasticity

Synaptic plasticity is the ability of neurons to either strengthen or weaken their connections in response to use or disuse and has been tightly associated with the foundation of memory and cognition. It was initially postulated by Hebb (1949) and was later biologically described by Bliss & Lømo (1973) who demonstrated that electrophysiological

stimulation to synapses caused prolonged activation. Biologically, synaptic plasticity involves remodeling of dendritic spines in neurons, affecting the efficiency of passing nerve impulses across the synapse (Abbott & Nelson, 2000). Long term potentiation (LTP) and long term depression (LTD) are two common forms of synaptic plasticity, where several studies in rodents have demonstrated enhancement of dendritic spines in LTP and loss or shrinkage in LTD, in coherence with retention of memory and performance in behavioral tests (Garín-Aguilar, et al., 2012; Keifer et al., 2015; Lai, Franke & Gan et al., 2012; Mahmmoud et al., 2015). Both LTP and LTD serve equally important functions, as strengthening and weakening of neural connectivity contributes to reshaping neurons and subsequently removal of undesired connections and maintenance of desired connections.

For neuronal reshaping to occur, they rely on proteins that have specific functions in modulating the synapse (Sutton & Schuman, 2006). Prior to addressing these proteins and how they are regulated by sleep, a description of plasticity will first be given.

1.12.1 Synaptic Plasticity in the Brain. An important component for initiating neuronal reshaping is influx of calcium ($Ca2^+$) into the postsynaptic neuron via glutamatergic N-methyl-D-aspartate (NMDA) receptors. When a neuron is in its resting state potential, NMDA cation channels are blocked by magnesium ($Mg2^+$) ions, restricting this $Ca2^+$ influx. This blockade may be removed, if there is a sufficient amount of action potentials from presynaptic neurons, which release glutamate that bind to glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors. This causes an influx of sodium (Na^+), which may sufficiently depolarize the postsynaptic neuron to an extent where it repels the $Mg2^+$ blockade and $Ca2^+$ may occur via the NMDA channels (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984). $Ca2^+$ influx is a crucial component in synaptic plasticity, as it may activate immediate early genes (IEGs) (Chowdhury et al., 2006; Sun & Lin, 2016; Van Haasteren, Li, Muda, Susini & Schlegel, 1999) that facilitate brain plasticity.

LTP is divided into an early phase (E-LTP) and a late phase (L-LTP). E-LTP lasts only for a few hours after activation and is *not* dependent on protein synthesis. It draws upon on IEGs that are already synthesized and stored in the synapse (Baltaci, Mogulkoc & Baltaci, 2019). If activation is prolonged in the synapse, it can potentially trigger L-LTP by innervating a second wave of genes that require de novo protein synthesis (Morgan & Curran, 1988). As described above, S6K1 and eIF4E are both crucial components in protein synthesis.

IEGs can be divided into two major classes; transcription factors that enhance or suppress genetic encoding at the transcriptional level, or proteins that directly regulate cellular functions (Sun & Lin, 2016). In this thesis, IEGs of interest are activity-regulated cytoskeleton-associated (Arc) proteins and neuronal PAS domain protein 4 (NPAS4) transcription factor. Also of interest, is the brain derived neurotrophic factors (BDNF). Their currently proposed functions in plasticity and relation to sleep, will be addressed below.

1.12.2 Sleep and plasticity. Sleep has been implicated in regulating synaptic plasticity. According to the theory of synaptic homeostasis, neuronal connections are strengthened (or potentiated) during wake and downscaled during sleep (Esser, et al., 2007; Tononi & Cirelli, 2006). During wakefulness, synaptic connections become stronger as we are constantly stimulated by, interact with and learn from our surroundings. Strong synaptic connections, however, require more use of energy and may lead to cellular stress. Moreover, high connectivity after wake might further reduce the formation of new dendritic spines. As synaptic connections are downscaled during sleep, it thus re-stabilizes synapses and returns to homeostasis (Tononi & Cirelli, 2014). In correspondence with process S of the two process model, synaptic downscaling is thought to be reflected in SWA, where highly synchronized slow waves reflect strong synaptic connections and firings between neurons. When sleep progresses into more desynchronized sleep, it has been postulated to reflect a weakening of synaptic connections (Esser, et al., 2007; Tononi & Cirelli, 2006).
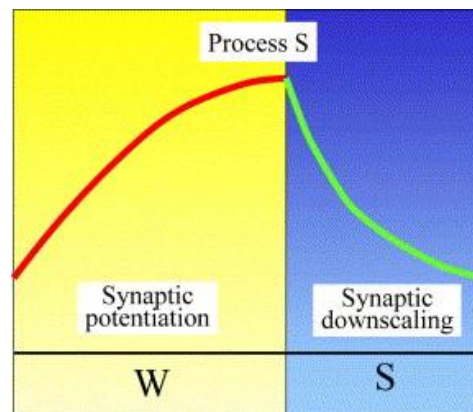
Figure 4. Model of synaptic homeostasis, taken from Tononi & Cirelli, (2006).

Sleep and homeostatic scaling has been demonstrated at the molecular and electrophysiological level. In a study by Maret, Faraguna, Nelson, Cirelli & Tononi (2011) they compared numbers of dendritic spines in the cortex of mice euthanized either after sleep in their rest phase, wake in their active phase or after sleep deprivation during their resting phase. It was found that animals euthanized after wake had higher numbers of spines and sleep deprived mice having even higher numbers. Supportive data has also been provided by Vyazovskiy, Cirelli, Pfister-Genskow, Faraguna, & Tononi (2008). They measured cortical and hippocampal glutamatergic AMPA receptors in rats, where they were more expressed following wakefulness, in comparison to sleep. Specifically, for the PFC, it has been demonstrated that excitatory postsynaptic potentials are increased after wakefulness and reduced after sleep (Liu, Faraguna, Cirelli, Tononi & Gao, 2010), indicative of potentiated synapses. To further elucidate how plasticity is affected by sleep, this thesis seeks to measure proteins that are known to be involved in regulating brain plasticity.

1.12.3 BDNF. BDNF is a neurotrophic factor which has an important role in neurogenesis (Binder & Scharfman, 2004), cell survival and regulation of synaptic plasticity (Lu et al., 2003). BDNF has been implicated in regulation of sleep homeostasis. In a study by

Faraguna et al. (2008), they performed unilateral BDNF injections into the frontal cortex of rats and found that SWA was elevated during NREM sleep in the same hemisphere. On the contrary, inhibition of the BDNF receptor TrkB reduces SWA. The authors further argue that these effects can be explained due to occurrence of synaptic potentiation. Similar findings have also been shown by Huber, Tononi & Cirelli (2007), where the BDNF was strongly correlated with amount of SWA following sleep deprivation, in addition to exploratory behavior in the open field test.

BDNF has also been studied in human subjects with the Val66Met polymorphism, which affects release of BDNF (Kuczewski, Porcher & Gaiarsa, 2010). Carriers of the Val66Met gene have been shown to have reduced SWA activity during SWS sleep in a regular night of sleep and after 40 hours of sleep deprivation. Additionally, their overall time spent in SWS was shown to be lower than in non-carriers of the Val66Met gene. Subjects with the Val66Met polymorphism also had reduced cognitive performance on the n-back task. (Bachmann et al., 2012). As the theory of synaptic homeostasis postulates with SWA reflecting strong connections between neurons, these findings may implicate that BDNF might have an important role in regulating synaptic connectivity and that it affects the ability of regulating sleep homeostasis.

Other studies have also examined BDNF levels following sleep deprivation. It has been shown that 6 hours of sleep deprivation in rats caused decreased levels of BDNF in the cerebellum and the brain stem, but not in the hippocampus (Sei, Saitoh, Yamamoto, Morita & Morita, 2000). This was also found by gentle handling sleep deprivation, where the hippocampus also was unaffected, whereas overall cortical levels of BDNF were elevated (Taishi et al., 2001). These elevated levels of cortical BDNF could further indicate that sleep dependent synaptic downscaling was reduced as the theory of sleep homeostasis postulates. Moreover, synaptic connections might be regulated differently across brain regions in relation

to sleep, which has already been proposed by Grønli et al., (2014). Liu et al. (2010) findings

of increased excitatory postsynaptic potentials following time in wake in the PFC, further

indicate that BDNF also is upregulated during wakefulness and prolonged wakefulness in this

region. It is also intriguing to speculate that potentially altered BDNF levels could be a

consequence of accumulated SWE during QW in rest workers or a consequence of reduced

attenuation of sleep pressure between work sessions (Grønli et al., 2017).

   1.12.4 Arc. The intracellular signaling following BDNF binding to the TrkB receptor

has been shown to trigger the IEG Arc. In a study by Kuipers et al. (2016), they studied Arc

translation by infusing BDNF in the rat dentate gyrus. Infusion of BDNF caused a potentiated

response alone. However, when injecting an Arc inhibitor prior to the BDNF infusion,

synaptic potentiation was blocked. This indicates that Arc possibly could act as an important

agent to carry out potentiation initiated by BDNF. Arc is also rapidly activated by $Ca2^+$ influx

into the postsynaptic membrane. It has been suggested that the main role of Arc in synaptic

plasticity, is reducing the surface expression of AMPA receptors, by internalizing them into

the cell via endocytosis (Chowdhury et al., 2006). Following activation of a neuron, it has

been shown that Arc accumulates the synapse and that it is negatively correlated with the

prominence of GluA1 AMPA receptors. It has therefore been postulated Arc plays a role in

inverse tagging, meaning that it balances out *excitatory* synapses by preventing the

enhancement of less desirable connections between neurons (Okuno et al., 2012).

   Arc has been found to positively correlate with REM sleep in rats (Grønli et al., 2012).

Relative to NREM sleep, REM sleep and wakefulness are brain states with elevated levels of

ACh signaling (Diekelmann & Born, 2010). In a study by Soulé et al. (2012), ACh agonists

were administered into the rat hippocampus, which increased Arc expression. As ACh is a

wake-promoting transmitter in the forebrain and also assumingly important for the initiation

of synchronized neuronal firings during REM sleep (Kayama et al., 1992; Van Dort et al.,

2015), it could therefore be that prolonged wakefulness, or increased REM sleep is due to ACh activity. In coherence, sleep deprivation studies in rodents have demonstrated that Arc protein levels are significantly upregulated in the rodent hippocampus following 6 hours of sleep deprivation (Terao et al., 2006). The same has also been shown for Arc mRNA in mice, in addition to Arc mRNA decreasing following 4 hours of recovery sleep (Thompson et al., 2010). It can thus be speculated increased wakefulness or REM sleep might trigger upregulation of Arc.

  1.12.5 NPAS4. NPAS4 is a more recently discovered IEG that functions as a transcription factor. Its activity is triggered by $Ca2^+$ influx following neural activity (Sun & Lin, 2016), such as Arc. Its role is to balance out the synapse, by increasing inhibitory GABAergic inputs that is released upon excitatory neurons. It is therefore thought that its main function is to maintain synaptic homeostasis, by stabilizing the ratio between GABAergic and glutamatergic connections in synapses (Lin et al., 2008). NPAS4 also affects the rate of BDNF mRNA expression. This, by enhancing transcription of BDNF mRNA, that may further increase BDNF protein expression, and go on to strengthen *inhibitory* synapses (Kim, Kim & Um, 2018).

  Due to the recent discovery of NPAS4, its relation to sleep is not much explored. One study who subjected rats to 96 hours of REM sleep deprivation via the flower pot method, found that it caused NPAS4 mRNA to be globally upregulated in the brain (Narwade, Mallick & Deobagkar, 2017). This could indicate that REM sleep is important for maintaining transcription of NPAS4 mRNA. Another study who subjected mice to 12 hours of sleep deprivation, found that NPAS4 mRNA expression was downregulated in the anterior cingulate cortex (Orozco-Solis et al., 2017). This may suggest that overall prolonged wakening overall reduces its expression. Comparing these studies is however difficult, as it cannot be excluded that NPAS4 is differently expressed throughout the brain. Both studies do

however indicate that NPAS4 transcription is affected by waking and sleep brain states. Therefore, the association between NPAS4 is not much explored. Especially not in relation the PFC. It is thus hard to indicate how NPAS4 might be affected during night work.

In sum, there are many indications of sleep-dependent regulation of protein synthesis at the level of translation and plasticity proteins. It seems that BDNF and Arc may have distinct associations with distinct types of sleep, where BDNF is associated with homeostatic NREM sleep processes and Arc with REM sleep and wakefulness. Thus, it might be that potential differences in these sleep stages could be indicators of compromised cognitive abilities in night shift workers. Due to NPAS4 being far less explored, its role in relation to sleep and wakefulness is more uncertain as studies has focused on NPAS4 at the transcriptional level.

1.13 Aims and Hypotheses

The overall aim of this thesis is to examine whether rats exposed to forced activity in either their circadian active phase (simulating human day shift) or resting phase (simulating human night shift), will show distinct changes in sleep and wakefulness parameters as measured by telemetric EEG and EMG transmitters. Moreover, if the sleep and wakefulness parameters will be associated with protein markers of translation and plasticity.

Firstly, I am to provide reliability for the rodent shift work model, by replicating previous findings of sleep and wakefulness (Grønli et al., 2017). Based on previous findings, I hypothesize that the rotating wheels will successfully keep the animals awake during the 8 hour work sessions. Across the work period, I further hypothesize that rest workers will have a steeper cumulative increase in SWE during QW compared to active workers, indicating a higher sleep drive. Since rest workers are in their home cages primarily during their active phase, I hypothesize that rest workers will spend more time in all sub-states of wakefulness

and less time in NREM/REM sleep relative to active workers. Moreover, that overall quality of NREM- and REM sleep will be impaired in rest workers. I hypothesize that impaired quality of sleep will be prominent by rest workers having shorter NREM- and REM sleep episodes, in addition to less SWA during NREM sleep. It is expected the opposite for active workers, as they are in their home cages primarily during their rest phase.

These findings will also be discussed in relation to human data, in order to evaluate the construct validity of the shift work model.

My second aim is to determine whether specifically sleep and wakefulness parameters can predict expression of translational- and plasticity proteins markers in the rat PFC. Based on Marti et al. (2017), I firstly hypothesize that the cap-bound translational marker pBMAL1 and non-cap bound pS6K1 will be downregulated in rest workers relative to their time-matched controls. No significant difference is expected for active workers relative to their time-matched controls. I further hypothesize that sleep quality may predict expression of peIF4E, although specifically which sleep parameters are yet to be examined due to a lack of research on how sleep is modulating this protein. For pS6K1, an association with sleep parameters is also yet to be investigated, especially as previous associations with sleep for this protein is conflicting (Tudor et al, 2016; Frolinger et al., 2018; Kam et al., 2019) and largely unexplored for the PFC. For plasticity markers, I hypothesize that BDNF will be upregulated in rest workers and unchanged in active workers. Here, I further hypothesize that homeostatic parameters of sleep (SWA in wakefulness, SWA in NREM sleep), sleep consolidation (NREM sleep episode durations) and overall time spent in NREM sleep will predict expression of BDNF. Arc expression is hypothesized to be reduced following rest work and unchanged after active work, as demonstrated in Marti et al. (2017). For Arc, I additionally hypothesize that REM sleep parameters (REM sleep episode length, number of REM sleep episodes and overall time spent in REM sleep) will predict Arc expression, as indicated in

other studies (Grønli et al., 2012; Kayama et al., 1992; Van Dort et al., 2015). Based on

Narwade et al. (2017), it is further hypothesized that NPAS4 will be upregulated in the PFC

following rest work and that REM sleep parameters will predict its expression. This is

however more uncertain, as research on this protein is not very extensive and not much

explored in relation to sleep.

2.0 Methods

2.1 Ethical approval

The experimental procedures were assessed and approved by the Norwegian Food

Safety Authority (permit number: 11321, appendix A). Procedures were performed according

to Norwegian laws and regulations regarding experimentation on animals (Forskrift om bruk

av dyr I forsøk, 2015) and the European Convention for the Protection of Vertebrate Animals

used for Experimental and other Scientific Purposes (Council of Europe, 1993). All the

experimenters involved were FELASA certified.

2.2 Animals and housing

Sleep data was collected from male (Sprague-Dawley, Ntac:SD, Taconic, Denmark)

rats (N = 16). On arrival from the supplier, they weighed around 300g and were housed in

groups of five in individually ventilated IV cages (480x375x210 mm, IVC system, Tecniplast,

Italy) at the university animal facility. After surgery, rats were housed individually in type III

cages (425x266x185mm) in the research group laboratory. Ventilation in the cages was 75 air

changes per hour, the temperature was maintained at 23 +/- 1ºC and humidity at 40% +/- 1%.

Food (rat and mouse no. 1, Special Diets Services, Witham, Essex, England) and water was

available ad libitum. Lighting conditions were organized in a 12:12 LD schedule. Lights-on

was set to 08:00 (zeitgeber time, ZT 0) with a one hour gradual transition from dark to light.

Lights were therefore fully lit at 09:00. Lights-off occurred at 20:00 (ZT 12) with a 1 hour

gradual transition to full darkness.

There was also a group that were kept undisturbed in their home cages (N = 16) serving as a time-matched control group for protein expression. Hence, these rats were not implanted with transmitters and did not provide any sleep data.

2.3 Surgery

Rats underwent surgery for the purpose of implanting EEG and EMG electrodes. Two types of transmitters were used, 4ET (*n=15*) and F40-EET (*n=1*) (Physiotel, Data Sciences International; St. Paul, MN).

For three days prior to surgery, the drinking water was administered antibiotics (trimethoprim, 0.16 mg/ml; sulfamethoxazole, 0.8 mg/ml; Bactrim, Roche; Basel, Switzerland). Before surgery, rats were anaesthetized by a subcutaneous injection (fentanyl, 0.227 mg/kg; fluanizone, 8.8 mg/kg; midazolam, 2.5 mg/kg; Hypnorm, Janssen Pharmaceuticals, Beerse, Belgium; Dormicum, Roche; Midazolam, Actavis, Teva Pharmaceuticals, Peta Tikva, Israel). EEG electrodes were placed frontal-parietal (FP, bregma coordinates: AP = 2.0 mm, ML = -2.0 mm; lambda coordinates: AP = 2.0 mm, ML = 2.0 mm) and frontal-frontal (FF, bregma coordinates: AP = 2.0 mm, ML = 2.0 mm; bregma coordinates: AP = 2.0 mm, ML = -2.0 mm). EMG electrodes were bilaterally implanted in the neck muscles. Transmitters were implanted in a subcutaneous pocket in the dorsomedial lumbar region (4ET) or in the neck region (F40-EET).

Immediately after surgery, ringer's acetate solution was intraperitoneally administered due to potential fluid loss during surgery (5 ml, Baxter). For the following two days, the animals were still administered antibiotics in their drinking water (trimethoprim, 0.16 mg/ml: sulfamethoxazole, 0.8 mg/ml; Bactrim, Roche). For three days after surgery, an analgesic was administered subcutaneously twice a day (buprenorphine; 0.3 mg/ml; Temgesic, Reckit & Benckiser; Slough, UK) and an anti-inflammatory treatment was administered once a day

(meloxicam; 5 mg/ml, Metacam, Boehringer Ingelheim, Germany). For 14 days after the surgery, the animals were allowed to recover. They received care every day throughout this period.

2.4 The Simulated Shift Work Model

Shift work was simulated by exposing the rodents to enforced ambulation for 8 hours a day in a motorized running wheel (Rat Running Wheel, TSE running wheel system, Bad Homburg, Germany, 24 cm diameter). Forced ambulation was centered either in the rats' resting phase (rest workers, RW, ZT 2-10), mimicking night shifts or centered in their active phase (active workers, AW, ZT 14-22) mimicking day shifts. Wheels rotated at 3 rounds per minute, adding up a total of 1.086 km moved per 8h work session. Food and water was available *ad libitum.* After each work session, the equipment was cleaned with a 5% ethanol solution and animals were returned to their home cages.

2.5 Design and Procedure

The experiment had an AB-design, where (A) baseline sleep and wakefulness data was first recorded for 24 hours in all animals (N = 15, one transmitter did not function). (B) Then the animals were randomly assigned to either the RW (*n = 9)* or AW (*n = 6*) condition and worked 3 consecutive shifts (RW worked ZT 2-10 and AW ZT 14-22). After the third work shift, animals were returned to their home cages for two hours before they were anesthetized by isoflurane in a sealed chamber and sacrificed by decapitation (RW sacrificed at ZT12 and AW at ZT0). The PFC was then sampled and frozen (-80 degree C^o) for later protein analysis. The undisturbed control groups were euthanized at ZT12 (*n = 8)* and ZT0 (*n = 8)* to provide time-matched protein data for RW and AW, respectively. Figure 5 below represents an overview of the experimental protocol.
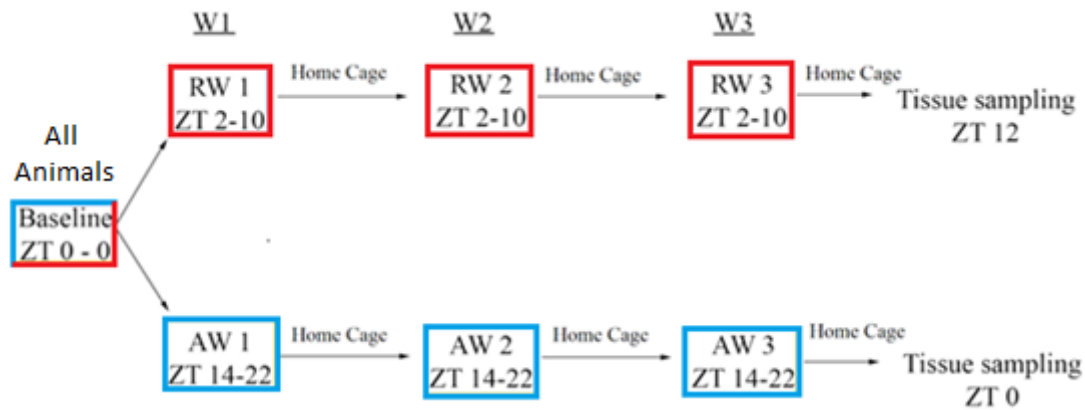
Figure 5. Experimental protocol. ZT = Zeitgeber, W = work, RW = rest work, AW = active work

2.6 Sleep Recording and Scoring

Sleep-wake data was recorded continuously at a sampling rate of 250 Hz for both the EEG and EMG signals (Dataquest A.R.T, version 4.1, Data Sciences International) throughout the experiment. Transmitter receivers (RPC-2, RPC-3, Data Sciences International) were placed beneath the animals home cage or next to the rotating wheels during work sessions. Different amplification from the transmitter signals (4ET: 240-fold; F40-EET: 200-fold) was corrected for by calibrating the receivers in Dataquest A.R.T.

Recorded data was scored offline in Neuroscore (version 2.0.1, Data Sciences International). Wakefulness, NREM and REM sleep were scored manually in epochs of 10 seconds. A sleep/wakefulness epoch was scored based on the characteristics of EEG and EMG recordings constituting $\geq 50\%$ of an epoch. For the purpose of sleep scoring, signals were filtered according to Neckelman & Ursin (1993), with an EEG low-pass filter set to 35 Hz on both FF and FP data, in addition to a high-pass filter set to 0.5 on the FF signal and 3.0 on the FP signal. Data used for following EEG power analyses were unfiltered.

2.7 Analysis of Sleep Data

Manually scored sleep data was processed in a customized Matlab based application, SLEEP Report, developed by Professor J. Wisor at Washington State University. The application effectively and reliably calculates sleep-wake parameters, such as minutes spent in desired sleep-wake states and EEG-power bands constituting the manually scored sleep stages. The application reports data in user-defined time intervals of the whole recording based on a desired number of sleep state epochs (i.e 8640 epochs = 24 hours). The dataset of thesis was set to 720 epochs per hour (2 hours), giving a total of 12 data points per 24 hours.

The script allows for re-classification of wakefulness into three substates; quiet wakefulness (QW), intermediate wakefulness and active wakefulness. These sub-states of wakefulness are re-scored based on peak-to-peak amplitude of EMG signals in epochs scored as wakefulness. In QW, the EMG peak-to-peak amplitude within an epoch of wakefulness is between 0 – 33%, intermediate wakefulness between 33% - 66% and active wakefulness EMG 66% - 100% (Grønli, Rempe, Clegern, Schmidt & Wisor, 2016).

The script further calculates SWA, based on average delta (1 – 4 Hz) power in the desired time interval. The term slow wave energy (SWE) refers the cumulative sum of SWA power across the sleep period. Gamma frequencies were defined within the range of 60 – 90 Hz.

2.8 Western Blot Analysis and m7GTP cap pull-down assay

Western blot is a technique used to separate and identify specific proteins in tissue. Here, the proteins are first separated through electrophoresis, where homogenized tissue is placed in agarose gel and separated by a surrounding electric field. Due to the proteins having different molecular weights, they will separate by moving different lengths. Specific primary antibodies are then added, which identifies and bind to the desired target proteins. Subsequently, secondary labeled antibodies are added. These bind to the primary antibodies

and can be visualized, giving indications of the magnitude of the specific proteins in the sampled tissue (Mahmood & Yang, 2012).

The antibodies used for visualizing the primary antibodies, were pS6K1 (1:1000, Santa Cruz Biotechnology #sc-7984), pBMAL1 (1:1000, Cell Signaling #13936), peIF4E (1:1000, Cell Signaling #9741), BDNF (1:200, Santa Cruz Biotechnology #sc-65514), Arc (1:500; Santa Cruz Biotechnology #sc17839) and NPAS4 (1:100, Santa Cruz Biotechnology #sc-293239).

2.9 Statistical analyses

All statistical analyses were run using Statistica (version 13.3, TIBCO Software incorporated). Repeated analysis of variance (rANOVA) was used to determine changes in sleep-wake parameters between RW and AW and across time intervals. The rANOVA analyses included one between-group variable to examine differences among RW and AW (group). Two within-group variables were used, one represented across the 2 hour time intervals (time) and the other as work days (Baseline, W1 and W2). Significant main and interaction effects (group x 2h intervals) were followed up with Fisher-LSD post-hoc tests. T-tests were also run on sleep-wake data, but only to examine differences between RW and AW in one 2 hour interval prior to euthanization. T-tests were also used for determining protein expression among RW and AW. A partial correlation analysis controlling for the animals group belonging was subsequently run. This was to more specifically determine the influence of sleep-wake parameters on protein levels in the PFC. Lastly, two different hierarchical regressions models were run. One was based on homeostatic variables and another on REM sleep and wakefulness variables (involved parameters elaborated in section 3.9). Both the correlation analysis and hierarchical regression were run on sleep and wakefulness data from two datasets. One was extracted from the 2 hour data prior to euthanization and the other from a 24 hour timespan ranging from the end of W2 to the end of W3. For all tests, statistical

significance was defined as all values within the range of $\alpha \leq .05$. Reported Cohen's d values follow the criteria of small-, medium- and large effect sizes, defined within the range of .20, .50, and .80, respectively (Cohen, 1988).

Differing degrees of freedom and sample sizes are due to lack of data. Reasons for this are some animals not exhibiting sleep-wake states in the intervals for the appropriate analysis, technical difficulties during data collection and unsuccessful extraction of some proteins from tissue. It should also be noted that data after W3 does only last for approximately 2 hours due to euthanization. Therefore, data following W3 is not included in all analyzes.

3.0 Results

Across the 24 hour baseline condition, all animals showed nocturnal patterns of wakefulness and sleep. Wakefulness occurred primarily during lights off (71% during lights off and 29% during lights on) and sleep during lights on (70% during lights on and 30% lights off). During baseline, there were no differences between the RW and AW, neither in QW ($F(1, 12) = 2.60, p = .133$), intermediate wakefulness ($F(1,12) = 1.17, p = .300$), active wakefulness ($F(1, 12) = 2.59, p = .133$), in NREM sleep ($F(1, 12) = 0.72, p = .412$) or in REM sleep ($F(1, 12) = 0.32, p = .581$). No differences were found in the length of NREM sleep episodes ($F(1, 12) = 9.25 , p = .356$) or REM sleep episodes ($F(1, 12) = 0.13, p = .732$).

3.1 Wakefulness During the Shifts

The enforced ambulation procedure successfully kept the animals awake during the work shifts, apart from a few micro sleep episodes. Descriptively, RW showed more sleep during their work hours than AW. Across the work hours at W1 to W3, RW slept in average 0.43 ± 0.14 minutes, 0.31 ± 0.14 minutes and 1.39 ± 0.51 minutes, a total average of $0.72 \pm$

0.20 minutes. For AW, the average minutes in sleep at W1 to W3 was 0.23 ± 0.15 minutes, 0.23 ± 0.15 minutes and 0.40 ± 0.20 minutes, with a total average of 0.29 ± 0.09 minutes.

Intrusion of sleep pressure in wakefulness during the shift work period was examined by calculating SWE in QW among RW and AW. No significant group effect was found during the 24 hours of baseline ($F(1, 9) = 1.62$, $p =.235$). From W1 to W3, the effect size was large ($d = 3.89$) between RW and AW, where RW increased by 404% and AW by 339%, indicating higher accumulation of sleep drive in RW. However, there was no significant group effect from W1 – W3 ($F(1, 9) = 1.94$, $p = .197$). Data from the analysis are shown in figure 6.
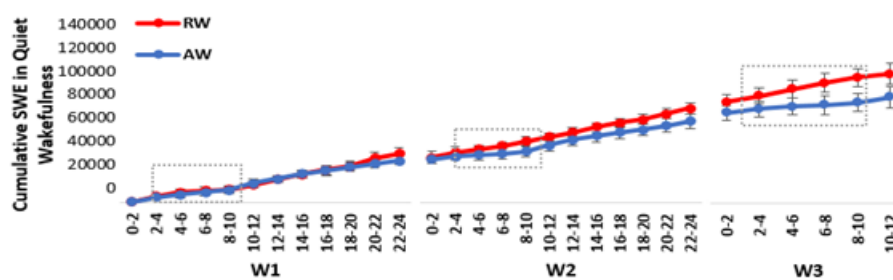
Figure 6. Cumulative SWE in QW during 3 day shift work period. Y-axes show cumulative SWE in QW and X-axes 2 hour intervals across shift work days. Stapled grey bars show when work occurred (Rest work ZT 2 – 10 and active work ZT 14-22). Each data point represents mean group SWE calculations. Data points indicate mean ± SEM.

During the three work sessions, time spent in QW and active wakefulness did not significantly differ between the groups ($p>.05$, group effects shown in table 1 of appendix B). For intermediate wakefulness, there was a significant interaction effect, showing that AW spent more time in this wakefulness state in the last half of W2 ($F(3, 39) = 3.79$, $p = .018$; all interaction effects shown in table 1 of appendix B). These data are shown in figure 7 below.
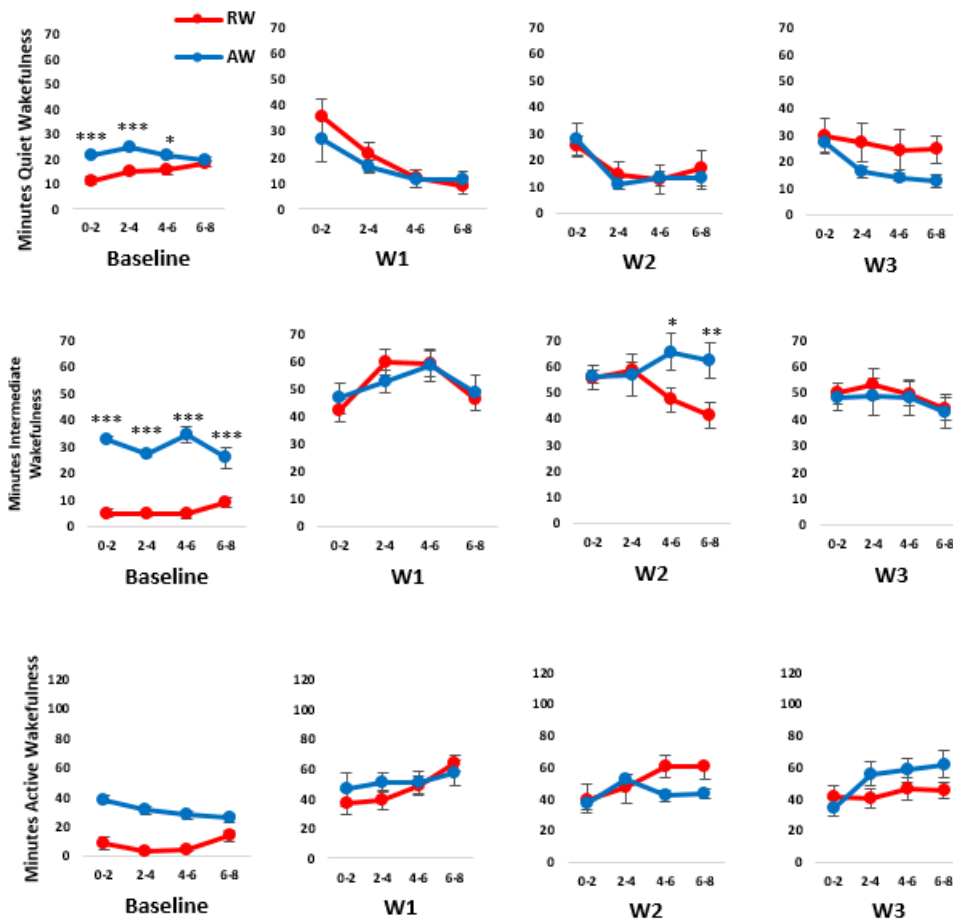
Figure 7. All sub-stages of wakefulness during work sessions and corresponding baseline

hours. X-axes represent 2 hour time intervals and y-axes minutes spent in wakefulness states

during respective time interval. Data points show mean ± SEM.

3.2 Waking in the 16 Hour Periods between Shift Work

Following shifts, animals were returned to their home cages and left undisturbed for

16 hours. In these 16 hours, total time spent in intermediate wake ($F(2, 26) = 40,79$, $p<.000$)

and active wake ($F(2, 26) = 14,83$, p<.000) was significantly reduced in both work groups

relative to their corresponding baseline recordings. QW was not significantly different from

baseline F(2, 26)=2,08, $p =.146$) (bar graphs in figure 8, 9 & 10).

There were significant interactions effects between group and the 2h intervals across

the work days, except from in active wake W2 (see table 2 in appendix B). Across the 16

hours between shifts, active workers had a larger tendency to spend more time in QW in the

first 2 hours after shifts. AW returned to their home cages for the last 2 hours of the active

phase (ZT10, dark) and RW during the last 2 hours of the resting phase (ZT22, light). This is

therefore likely to represent wakefulness driven by their endogenous circadian rhythm. In the

subsequent 12 hours (darkness/active phase for RW and light/rest phase for AW) RW tended

to spend more time in especially intermediate wakefulness, relative to AW. In the last 2 hours

before the next shift (light for RW, dark for AW), active workers became more awake than

rest workers. This was especially prominent prior to both shifts in QW and before W2 in

intermediate- and active wakefulness.

The total amount of time in each wakefulness state did however remain largely the

same between the groups across all work days. After W2, there was a significant difference

between the groups, where RW spent more time in intermediate wakefulness. It was however

very modest difference (bar graphs 9c).
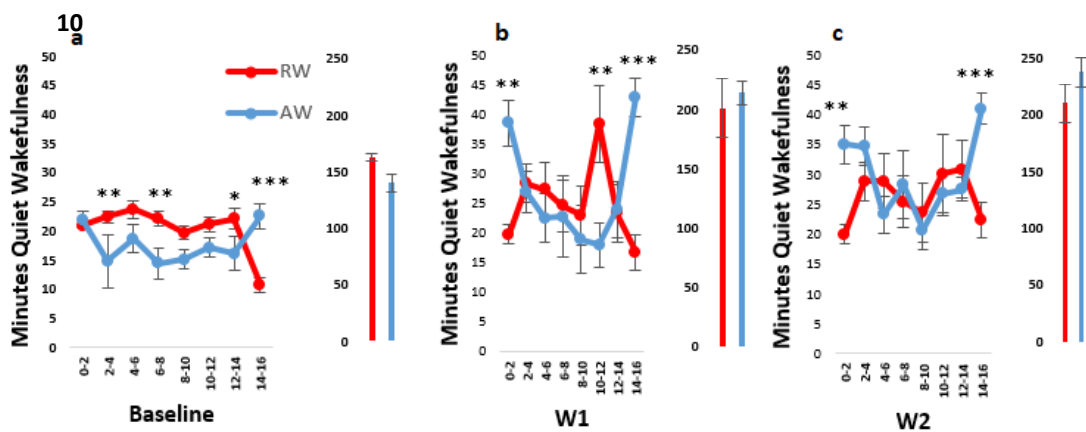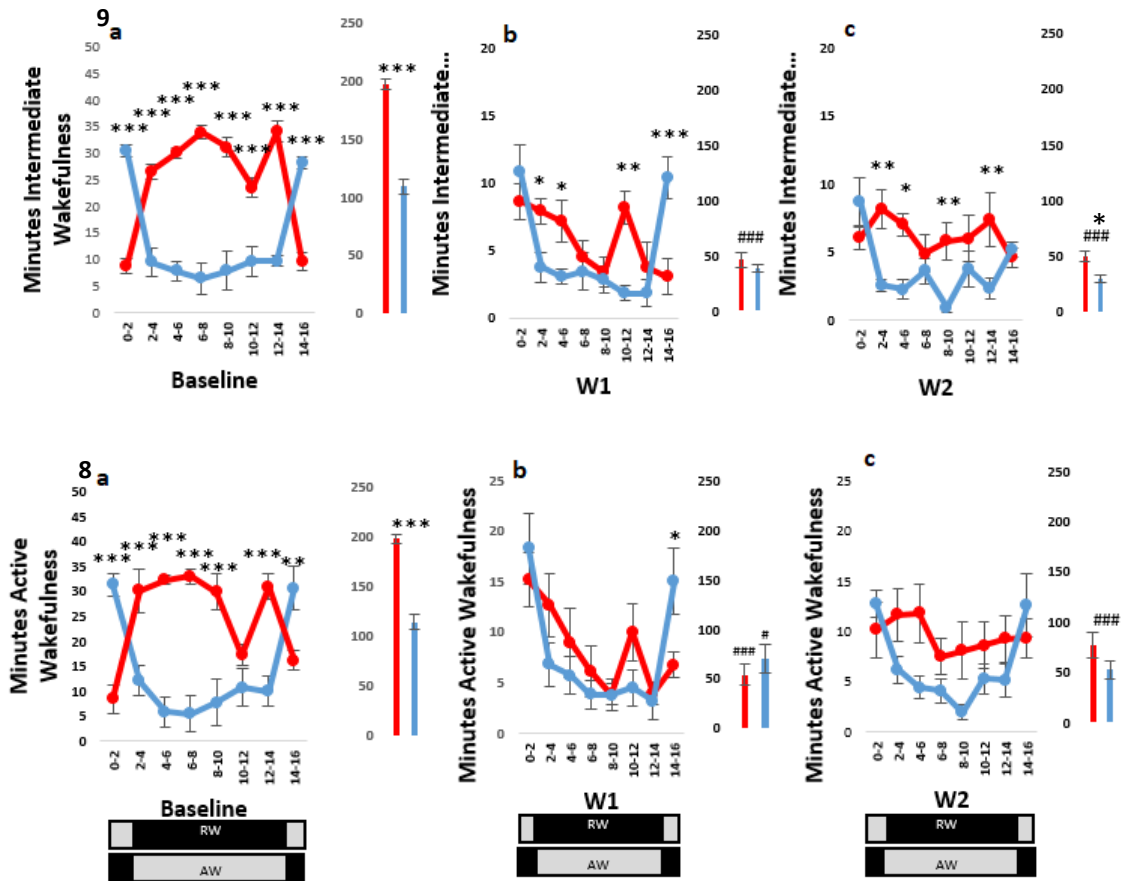
Figure 8a – 10c. Waking during 16 hours between work shift 1 and 2 and corresponding

hours in baseline. Y-axes represent minutes in waking states and x-axes the time course in 2

hour intervals. Each data point show group means ±SEM for each time interval. Bars to the

right in each figure, show group means ±SEM of total minutes spent in respective

wakefulness state. Bottom bars show light:dark conditions for rest workers and active workers

across baseline and shift work measurements. W = Work. Between-Group differences

denoted at * p<.05, **p<.01, *** p<.001 and significant difference relative to baseline

measurement at # p<.05, ## p<.01 and ### p<.001.

3.3 Sleep in the 16 Hour Period between Shift Work

In the 16 hours between shifts, both groups increased their total time spent in NREM

sleep relative to their corresponding circadian timed sleep in the baseline measurement. A

significant difference was prominent after W1 but not after W2 ($F(2, 26) = 4.46$, $p = .022$).

RW also increased their total time in REM sleep after both W1 and W2, relative to their baseline. This was however not seen in AW ($F(2, 26) = 16.84$, $p<.000$) (Bar graphs figure 11&12 b/c).

There were significant interaction effects across the 2 hour time intervals (see table 2 in appendix B). In the 2 hours after the shifts, RW tended to spend more time in NREM sleep. In the following 12 hours however, AW tended to spend more time in NREM sleep compared to RW. In the last 2 hours before the onset of the next shift, RW then spent more time in NREM sleep. Notably, this pattern is the opposite of what is reported during wakefulness above. In REM sleep, a significant interaction effect was seen after W1 but *not* after W2. This was however a very modest difference, with RW only having decreased time in REM sleep during the 10-12 hour interval after W1 (figure 11b).
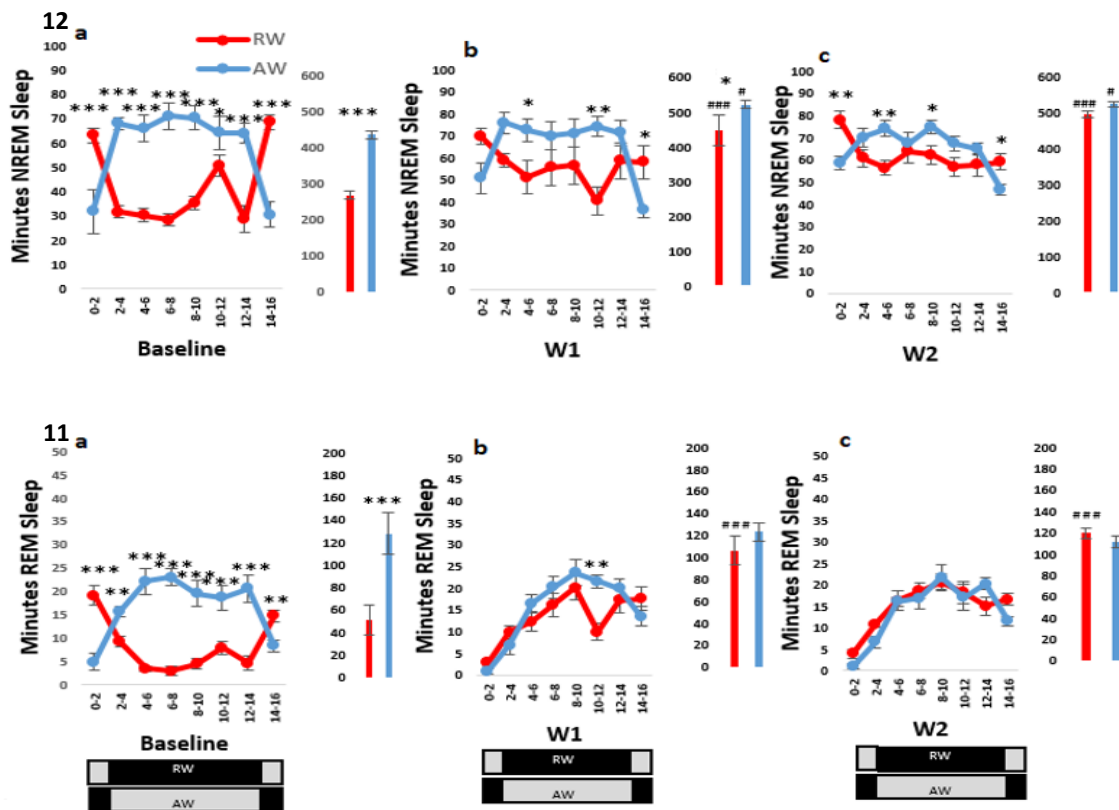
Figure 11a – 12c. Sleep in the 16 hours between work shift 1 and 2 and corresponding baseline hours. Y-axes represent minutes spent in NREM/REM sleep and x-axes the time course in 2

hour intervals. Data points show group means ±SEM of total minutes spent in NREM/REM sleep for each interval. Bars to the right in each figure, show group means ±SEM of total minutes spent in sleep states. Bottom bars show light:dark conditions for rest workers and active workers across baseline and shift work measurements. W = Work. Between group differences denoted at *p<.05, **p<.01, ***p<.001 and significant difference relative to baseline measurement at # p<.05, ## p<.01 and ### p<.001.

3.4 Consolidation of Sleep During the Shift Work Period.

Consolidation of sleep was examined using the length of NREM- and REM sleep episodes in the 16 hours following W1 and W2. For between-group effects, NREM sleep episode lengths tended towards significance with a large effect size ($F(1, 12) = 3.43$, $p = .089$, $d = 1.21$). REM sleep episode lengths were not significant and had a small effect size ($F(1, 12) = 0.25$, $p = .623$, $d = 0.32$). Data is summarized in figure 13 below.

During baseline, average duration of NREM sleep episodes were similar among the groups (RW = 170.04 seconds ± 7.00; AW = 182.56 seconds ± 9.39). NREM sleep episode durations were very modestly affected in AW after W1 (182.16 seconds ± 19.59) and W2 (179.08 seconds ± 19.70). RW, however, showed more marked changes in NREM sleep episode durations after both W1 (135.69 seconds ± 14.60) and W2 (146.20 seconds ± 14.68). Therefore, consolidation of NREM sleep seemed to be impaired in RW relative to AW.

For REM sleep episode lengths, both groups had similar values in baseline (RW = 109.27 seconds ± 4.32; AW = 104.33 seconds ± 5.80). Duration of REM sleep episodes were reduced in both groups after W1 (RW = 79.44 seconds ± 9.25; AW = 98.41 seconds ± 12.41) and W2 (RW = 86.43 seconds ± 5.69; AW = 84.78 seconds ± 7.63). The average NREM sleep episodes did not largely differ between the groups. Therefore, RW and AW did not seem to be

differently affected by work in consolidation of REM sleep.
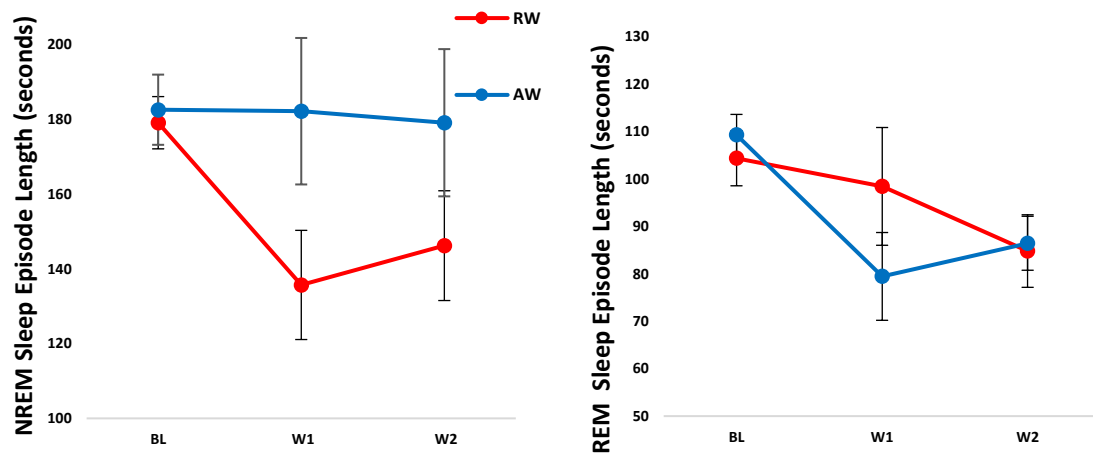
Figure 13. NREM- and REM sleep episode lengths in baseline (BL) and 16 hours following W1 and W2. Data points show mean ± SEM.

3.5 Homeostatic Sleep Drive between Shifts

To measure homeostatic sleep drive as a consequence of type of shift work, SWA activity in sleep between the shifts was examined. There was a gradual decline in SWA during NREM sleep in both RW and AW, indicating attenuation of the sleep drive in both work groups. After W2, the amount of SWA was significantly different between the groups ($F(7, 84) = 2.88$, $p = .010$), where RW showed reduced SWA the first 6 hours after the ended shift, compared to AW (Figure 14). There was no significant interaction after W1 ($F(7, 84) = 0.84$, $p = .561$).
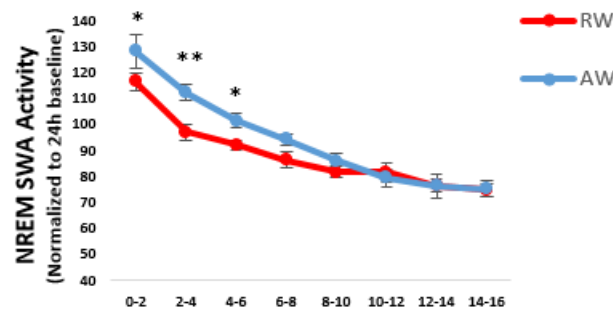
Figure 14. Slow wave activity (SWA) activity during NREM sleep after W2. Y-axes show

SWA normalized to the 24h baseline condition and x-axes 2 hour intervals across the 16

hours. Each data point show group means for a respective interval. Vertical thin lines show

SEM ±. Between-group differences denoted at *p<.05, **p<.01, ***p<.001.

3.6 Sleep and Wakefulness in the 2 Hours before Euthanization

Differences in sleep and wakefulness patterns were also addressed in the last two

hours prior to euthanization. The main purpose for this analysis is to examine in later analyses

how sleep-wake parameters may predict expression of translation- and plasticity markers.

The effect size was medium to large between RW and AW for time spent in the states

of wakefulness after W2, however the effect was not significant, QW ($t(11) = -1.82$, $p = .097$,

$d = 1.22$), intermediate wake ($t(11) = -.96$, $p = .358$, $d = 0.64$) and active wake ($t(11) = -1.23$,

$p = .242$, $d = 0.86$).

RW did not spend more time in NREM sleep than AW ($t(11) = 1.10$, $p = .294$, $d =$

.74), but the amount of REM sleep was significantly higher ($t(11) = 3.58$, $p = .004$, $d = 2.50$).

Graphs and a table summarizing the results are shown in table 3, figure 1 and 2 of appendix

B.

3.7 Cortical Expression of Translation and Plasticity markers Compared Time-Matched Controls.

3.7.1 Change relative to time-matched controls in translational markers.

Following rest work, the cap-bound proteins pBMAL1 ($t(13) = 3.19$, $p = .007$, $d = 1.65$) and peIF4E ($t(10) = 2.56$, $p = .028$, $d = 1.48$) were significantly downregulated in the PFC (-52.38% ± 0.21; -60.82% ± 0.16, respectively). pS6K1 ($t(12) = 1.96$, $p = .073$, $d = 0.98$) was also downregulated (-35.84% ± 0.12) and had a strong tendency towards significance. All effect sizes were large in RW.

Following active work, the cap-bound proteins pBMAL1 ($t(12) = -0.90$, $p = .386$, $d = 0.48$) and peIF4E ($t(10) = -0.11$, $p = .916$, $d = 0.05$) were largely unchanged (25.15% ± 0.19; 4.42%, ± 0.30, respectively), as they were not significant and had small effect sizes. pS6K1 ($t(11) = 3.21$, $p = .008$, $d = 1.80$) was significantly upregulated with a large effect size in AW (94.75%, ± 0.39). Results summarized in figure 15 below.
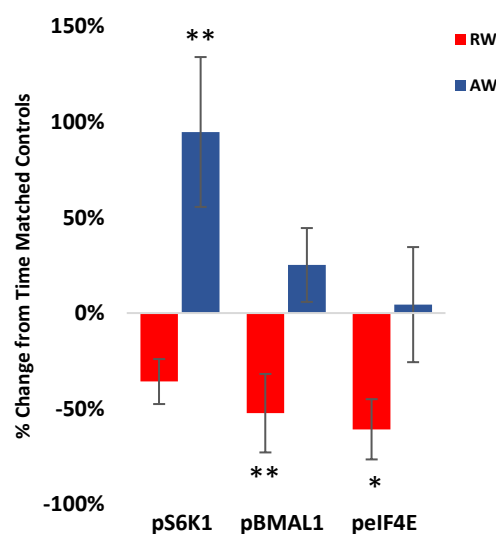
Figure 15. Percent change in RW and AW translational protein markers, relative to their time-matched controls. 0% represent average protein levels of controls. Vertical thin lines show ±SEM. Significant difference from time-matched-controls denoted at * p<.05, ** p<.01.

3.7.2 Change relative to time-matched controls in plasticity markers. Following work, BDNF was unaffected in both RW ($t(15) = -0.16$, $p = .880$, $d = 0.08$) and AW ($t(12) = -0.02$, $p = .984$, $d = 0.01$). After rest work, Arc ($t(12) = 2.21$, $p = .047$, $d = 1.18$) was significantly reduced with a large effect size (-55.12% ± 0.41). NPAS4 was upregulated in RW (71.66% ± 26) but was however not significant with a medium effect size ($t(15) = -1.37$, $p = .192$, $d = 0.68$).

Following active work, Arc ($t(11) = 0.27$, $p = .795$, $d = 0.15$) and NPAS4 ($t(12) = 0.75$, $p = .470$, $d = 0.40$) was not significantly affected with small effect sizes (-10.41% ± 0.28; 23.32% ± 0.11, respectively). Results summarized in figure 16 below.
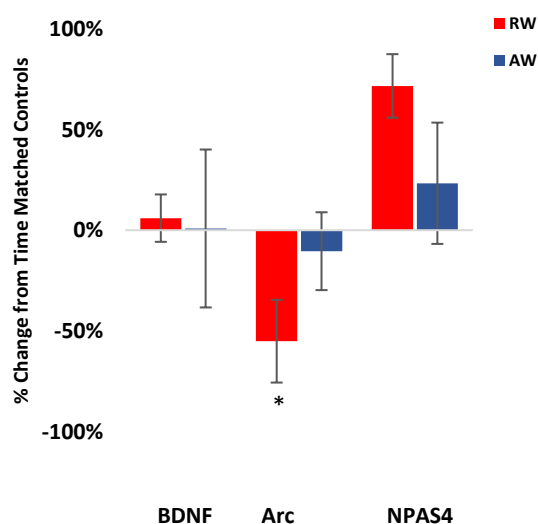
Figure 16. Percent change in RW and AW plasticity markers, relative to their time-matched controls. 0% represent average protein levels of control animals. Vertical thin lines show ± SEM. Significant difference from time-matched-controls denoted at * p<.05

3.8 Does Sleep and Wakefulness predict changes in Translation and Plasticity markers?

Based on the high individual variance in the sampled PFC proteins, it was sought to examine whether these changes could be seen in coherence with observed sleep-wake parameters. The first analyses were based on the 2 hour data prior to euthanization. See correlation matrix in figure 2a, along with sample sizes and p-values in table 4 of appendix B. In the second analyses, I tested if the last 24 hours of sleep-wake behavior could predict protein expressions. These data are shown in figure 2b, in addition to sample sizes and p-values in table 5 of appendix B.

Overall findings were that expression of several translational and plasticity markers were associated with the homeostatic sleep drive observed in QW and NREM sleep parameters. The plasticity marker NPAS4, was on the other hand associated with number of wakefulness episodes.

Specifically, pS6K1 was not associated with NREM sleep the last 2 hours ($r = .19$, $p =.767$) but showed a negative correlation with total time in NREM sleep in the last 24 hours ($r = -.58$, $p = .037$). The cap-bound peIF4E protein, showed a strong positive correlation with length of the NREM sleep episodes in the last 2 hours ($r =.91$, $p =.002$), but not in the last 24 hours ($r =.17$, $p =.646$). For cap-bound pBMAL1, no significant correlations were found ($p>.05$).

For plasticity markers, BDNF exhibited a strong positive correlation with SWA in QW in both 2 hour and 24 hour data ($r = .73$, $p = 0.17$ and $r = .60$, $p = .038$, respectively). In the 24 hour data, BDNF was also strongly correlated with NREM sleep episode length ($r =.90$,

p<.001), in addition to a strong negative correlation with number of NREM sleep episodes (*r* =-.82, *p*<.001). Associations between BDNF and NREM sleep episode length and number were not present in the 2 hour data (*r* =-.09, *p* = .802; *r* =-.30, *p* =.362, respectively). Lastly, there was a negative correlation between NPAS4 and number of wakefulness episodes in the 24 hour data and 2 hour data (*r* =-.53 *p* =.049; *r* =-.56, *p* =. 059).

3.9 Work and sleep (homeostatic drive, consolidation and quality) predict different aspects of Translational and Plasticity Markers in the PFC

In the next step, I aimed to investigate if type of work or sleep could predict the expression of translation and plasticity markers by using step-wise hierarchical regression analyses.

One regression model was based on type of work and NREM sleep parameters. This model was run on pS6K1, pBMAL1, peIF4E and BDNF, as they correlated with NREM sleep parameters. Work (RW or AW) was used as step 1, homeostatic sleep drive (SWA in QW) as step 2, sleep consolidation (NREM sleep episode length and total minutes in NREM sleep) as step 3 and sleep quality (SWA in NREM sleep) as Step 4. Data from the 2 hours are summarized in table 1 and 24 hours data in table 2 below.

3.9.1 Work predicts expression of pS6K1, pBMAL1 and peIF4E. Type of work strongly predicted pS6K1 expression in the 2 hour and 24 hour data (*adj. R^2* =.85 and *adj. R^2*= .73, respectively). All steps tested for pS6K1 reached significance (*p*<.005), but only very modest changes were made to adj. R^2 across the remaining steps. The same tendency was seen for pBMAL1, where work was the major predictor in both the 2 hour and 24 hour data (*adj. R^2* =.39 and *adj. R^2* =.39, respectively). Work provided some predictive power for peIF4E in both the 2 hour and 24 hour peIF4E data. However, adding more steps reduced adj. R^2 in the 24 hour peIF4E data. This shows that work was the major predictor for pS6K1, pBMAL1, and

also for peIF4E when examining sleep data in a 24 hour time window. For BDNF

interestingly, no predictive power was provided by work (see step 1, table 1 & 2).

3.9.2 Homeostatic sleep drive predicts expression of BDNF. Adding SWA in QW

enhanced the predictive power for BDNF in 2 hour and 24 hour data (*adj. R^2 change* = .45 and

adj. R^2 change = .24, respectively). Interestingly, adding the other NREM sleep parameters for

the 2h data reduced the predictive power of sleep drive on BDNF expression, suggesting that

acute sleep pressure is one important parameter for the expression of cortical BDNF. The

model including work and homeostatic sleep drive was significant (p = .037, see step 2 in

table 1).

3.9.3 Sleep consolidation predicts expressions of peIF4E and BDNF. Sleep

consolidation enhanced the predictive power of peIF4E in the 2 hour data by increasing adj.

R^2 by .58 and by .52 in the 24 hour BDNF data. Both models were significant (p<.003). This

suggests that sleep consolidation is a parameter involved in regulating peIF4E and BDNF

expression (see step 3 table 1 & 2).

3.9.4 Sleep quality did not predict expression of any proteins. The addition of sleep

quality in the last step, did not provide any marked predictive power for any of the proteins.

(step 4, table 1 & 2).

Table 1

Predicting protein markers of translation and plasticity last 2 hours of NREM sleep and

wakefulness parameters.

	pS6K1	pBMAL1	peIF4e*	BDNF
Step 1, work	$F(1,11)=72.39$	$F(1, 11)=8.68$	$F(1,7)=3.98$	$F(1,10)=0.68$
p	<.001	.013	.09	.430
RW or AW				
R^2	.86	.44	.36	.06
Adj. R^2	.85	.39	.27	.00
Step 2, Sleep drive	$F(2,9)=42.67$	$F(2,9)=3.82$	-	$F(2,8)=5.12$
p	<.001	.063	-	.037
SWA in QW				
R^2	.90	.45	-	.56
Adj. R^2 (change)	.88 (.03)	.34 (-.05)	-	.45 (.45)
Step 3, Sleep consolidation	$F(4,7)=18.49$	$F(4,7)=2.51$	$F(2,6)=23.44$	$F(4,6)=2.24$
p	<.001	.140	.002	.181
Duration of NREM sleep episode				
Total minutes in NREM sleep				
R^2	.91	.58	.89	.59
Adj. R^2 (change)	.86 (-.02)	.35 (.01)	.85 (.58)	.33 (-.09)
Step 4, Sleep quality	$F(5,6)=12.98$	$F(5,6)=2.42$	-	$F(5,5)1.52$
p	.003	.156	-	.330
SWA in NREM sleep				
R^2	.92	.67	-	.60
Adj. R^2 (change)	.84 (-.02)	.39 (.04)	-	.21 (-.11)

*Note: *pEIF4e regression only contained two-steps due to initial 4-step model overfitting.*

Work used as step 1 and NREM sleep episode length as step 2.

Table 2

Predicting protein markers of translation and plasticity from last 24 hours of NREM sleep

and wakefulness parameters.

	pS6K1	pBMAL1	peIF4E*	BDNF
Step 1, work	$F(1,14)=42.77$	$F(1,14)=10.53$	$F(1,10)=3.83$	$F(1,13)=0.00$
p	<.001	.006	.060	.985
RW or AW				
R^2	.75	.43	.28	.00
Adj. R^2	.73	.39	.20	.00
Step 2, Sleep drive	$F(2,11)=16.90$	$F(2,11)=4.94$	-	$F(2,10)=2.85$
p	<.001	.029	-	.105
SWA in QW				
R^2	.75	.47	-	.36
Adj. R^2 (change)	.71 (-.02)	.38 (-.01)	-	.24 (.24)
Step 3, Sleep consolidation	$F(4,9)=16.63$	$F(4,9)=3.60$	$F(3,7)=1.03$	$F(4,8)=10.61$
p	<.001	.051	.440	.003
Duration of NREM sleep episode				
Total minutes in NREM sleep				
R^2	.88	.59	.30	.84
Adj. R^2 (change)	.82 (.11)	.40 (.02)	.00 (-.20)	.76 (.52)
Step 4, Sleep quality	$F(5,8)=11.83$	$F(5,8)=3.71$	-	$F(5,7)=8.89$
p	.002	.049	-	.006
SWA in NREM sleep				
R^2	.88	.61	-	.86
Adj. R^2 (change)	.80 (-.02)	.45 (.05)	-	.76 (.00)

*Note: *pEIF4e regression only contained two-steps due to initial 4-step model overfitting.*

Work used as step 1 and NREM sleep episode length as step 2.

3.10 Work, REM sleep and wakefulness parameters predict different aspects of Arc and NPAS4 in the PFC

Another regression model was run for NPAS4 and Arc, as NPAS4 correlated with wakefulness episodes and Arc has been postulated to be affected by cholinergic activity during REM sleep (Van Dort et al., 2015; Soulé et al., 2012). This model was based on type of work, wakefulness and REM sleep parameters, Work (RW or AW) was used as step 1, REM sleep consolidation (amount of REM sleep episodes and total time in REM sleep) as

step 2 and sleep fragmentation (number waking episodes) as step 3. These analyses are summarized in table 3.

3.10.1 No strong predictors of Arc expression after simulated shift work.

The full model of work, REM sleep and waking frequency was not a good fit for the 2 hour Arc data, as adj. $R^2 = 0$ (Step 3, table 3). For the 24 hour sleep-wake data, REM sleep consolidation provided some predictive power for the model (*adj. R^2 = .22*). However, none of the steps were significant, indicating that the overall model was not a good fit for the Arc protein.

3.10.2 Number of awakenings predicts expression of NPAS4. Type of work did not provide any predictive power for NPAS4 2 hour data (*adj.R^2 =. 00*) and modestly for 24 data (*adj.R^2 = .12*). Number of awakenings during the 2 hours prior to euthanization enhanced adj. R^2 by .46 and showed a strong tendency towards significance (*p* = .060). Adding REM sleep did not provide any predictive power. This suggests that waking frequency is associated with NPAS4 expression. In the 24 hour data, only modest increases in predictive power were obtained across all steps. None of the steps were significant suggesting that the 24 hours data did no predict the expression of NPAS4.

Table 3

Predicting plasticity markers from REM sleep and wakefulness parameters.

	Arc (2 hour)	Arc (24 hour)	NPAS4 (2 hour)	NPAS4 (24 hour)
Step 1, work	$F(1,8)=1.76$	$F(1, 11)=2.16$	$F(1,11)=0.91$	$F(1,14)=3.00$
p	.221	.170	.360	.105
RW or AW				
R^2	.18	.16	.07	.18
Adj. R^2	.08	.09	.00	.12
Step 2, REM sleep consolidation	$F(2,7)=0.66$	$F(3,8)=2.61$	$F(3,9)=0.77$	$F(3,11)=0.83$
p	.547	.123	.540	.504
REM sleep episode amount				
Total time in REM sleep				
R^2	.16	.49	.21	.18
Adj. R^2 (change)	.00 (-.08)	.31 (.22)	.00 (.00)	.00 (-.12)
Step 3, Waking frequency	$F(3,6)=0.44$	$F(4,7)=1.75$	$F(4,8)=3.51$	$F(4,10)=1.89$
p	.744	.242	.060	.188
Number waking episodes				
R^2	.17	.50	.64	.43
Adj. R^2 (change)	.00 (.00)	.21 (-.10)	.46 (.46)	.20 (.20)

4.0 Discussion

This thesis has simulated human night work by subjecting rats to forced activity in automatically rotating wheels for 3 consecutive days. Rest workers (mimicking night work) were exposed to 8 hours of forced activity during their rest phase and active workers (mimicking day work) during their active phase. Rest workers thus had to stay awake in time windows when they were physiologically primed for sleep and recover sleep in time windows when they were physiologically primed for wakefulness.

The first aim was to replicate previous sleep and wakefulness findings in the simulated shift work model (Grønli et al., 2017). The second aim was to determine whether specifically sleep and wakefulness parameters could predict protein markers of translation and synaptic plasticity underlying cognitive functioning. Results showed that previous sleep, wakefulness and protein data from the shift work model were replicated. In addition, that sleep and

wakefulness parameters predicted protein expression in the PFC. Below, I will firstly discuss the sleep and wakefulness findings in relation to existing human and animal literature. Secondly, I will address a poorly understood topic, how sleep and wakefulness parameters may predict protein expression in the PFC. An evaluation of the shift work model of will lastly be addressed.

4.1.1 Rest Work Increases Homeostatic Sleep Drive in Wakefulness

The ideal waking state consists of high frequency EEG activity (Berry et al., 2012). When the intrusion of SWA becomes prominent during wakefulness, it represents that a drive towards sleep is high (Finelli et al., 2000). The presented data indicates that rest workers had an increased drive towards sleep during wakefulness, relative to active workers. This is firstly supported by our data showing that rest workers had a steeper incline of SWE during QW across the work period, relative to active workers. Secondly, that rest workers slept more during the work shifts.

Although behavioral data was not collected, the behavioral consequences of an increased sleep drive during wakefulness has been studied by other authors. During prolonged wakefulness, the likelihood of neurons suddenly going "offline" is represented by an increase in SWA, which has been associated with reduced performance in the sugar pellet reward task (Vyazovskiy et al., 2011). SWA during waking has moreover been shown to predict increased PVT reaction times in rodents (Walker, et al., 2011). In humans alike, PVT reaction times also increases over the course of a sleep deprivation period, which has been associated with lower EEG frequencies indicative of increased sleep drive (Tian et al., 2018). In a naturalistic night work setting, this is likely to be connected with falling asleep behind the steering wheel (Åkerstedt, 2019; Finkelman, 1994) and errors among health care personnel (Johnson et al., 2014). The present data therefore indicates that the activity pattern of night workers more

rapidly increases EEG power band frequencies associated with reduced cognitive functioning, resulting in a reduced vigilance state.

Neurobiologically, QW has been linked reduced wake-promoting histaminergic activity in the posterior hypothalamus, relative to in intermediate and active wakefulness (Takahashi, Lin & Sakai, 2006). Increased SWA during quiet waking, additionally represents an intrusion of sleep-promoting GABAergic neurons of the anterior hypothalamus (Scammel et al., 2017). The combination of quiet waking and SWA in the EEG signals, therefore represents a brain state in where a drive towards sleep very high and cognitive lapses may occur. Thus, neurobiological data supports an argument of our results representing an impaired vigilance state in night workers.

4.1.2 Time in NREM Sleep Depends on Type of Work.

When the animals were in their home cages between work shifts, rest workers spent more time awake and less time in restorative NREM sleep. The two-process of sleep regulation (Borbèly, 1982) proposes that the *demand* for recovery sleep depends on prior activity and that its *opportunity* depends on the time of the day (Daan et al., 1984). Demand was kept the equal for the groups, as they both worked 8 hour shifts and were in their home cages for 16 hours. For opportunity, rest workers returned to their cages when their circadian rhythm was rising. On the contrary, the circadian rhythm of active workers was declining. Therefore, when returning from a night shift, it resulted in less time asleep due to having a lower drive towards sleep. It is evident that sleep and wakefulness patterns were affected by circadian factors, as time spent in both NREM sleep and wakefulness changed in accordance with the light and dark conditions. Grønli et al., (2017) further showed that either constant darkness or a regular 12:12 light-dark condition affected recovery sleep after a 4 day shift work protocol. This further strengthens that circadian factors are an overlying variable

resulting in loss of sleep across a night shift period. Polysomnographic data from human night workers show that time in SWS remain unchanged (Matsumoto, 1978; Torsvall et al., 1989; Foret & Benoit, 1974) or reduced (Åkerstedt et al., 1991) after night shifts. The presented data cohere with these findings, by experimentally demonstrating that sleep opportunity in a night work context affects the overall potential of spending time in restorative NREM sleep.

4.1.3 NREM Sleep Quality and Episode Lengths of Depend on Type of Work

In addition to rest workers spending less time in NREM sleep, its quality was also reduced. In the first six hours after the second shift, animals returning from active work had significantly higher SWA during NREM sleep than the group returning from rest work. This may on one hand seem contradictory, as it is aforementioned addressed that rest workers had a higher drive towards sleep. A higher sleep drive may result in increased SWA during NREM sleep, which is often reported observed after sleep deprivation (Borbèly & Neuhaus, 1979; Tobler & Borbèly, 1990). These data do therefore further demonstrate that a rising circadian rhythm reduces NREM sleep quality and thus a restorative property of NREM sleep. Human polysomnography recordings have shown that SWA is unaffected in day time NREM sleep following night shifts (Åkerstedt et al., 1991). As well as experimentally, in morning relative to night time recovery sleep following prolonged wakefulness (Parrino et al., 1993). Although our data show reduced SWA and not unchanged, it does however still indicate that sleep opportunity in a night work context impairs NREM sleep quality.

The results further indicated that NREM sleep episodes were shorter in rest workers, compared to active workers. This was prominent across the whole work period, where rest workers had shorter sleep episodes after both shifts. Sleep continuity is alike SWA a function of prior time awake, where increased SWA after sleep deprivation normally reduces awakenings in recovery sleep (Borbély & Tobler, 2011; Dijk & Von Schantz, 2005). As

addressed above, our data showed that rest workers had reduced SWA in NREM sleep following work. This may further strengthen that their sleep opportunity and not their prior time in wakefulness affected their NREM sleep episode length. It has been argued that NREM sleep episode lengths are primarily circadian driven. This is based on active phase sleep having a strong drive towards wakefulness, causing shorter episodes of NREM sleep and more awakenings. The opposite is true for rest phase sleep, where the drive is stronger towards sleep (Rempe et al., 2018). These findings cohere with previous reports of night workers having fragmented sleep (Chang & Li, 2019), which suggests that daytime sleep after night work negatively impacts sleep consolidation.

Taken together, the length, quality and consolidation of sleep are all three important parameters that affects its restorative functions (Dijk & Von Schantz, 2005). When returning from rest work, all the aforementioned restorative functions of NREM sleep were reduced in rest workers. Night work therefore results in a vicious circle, making it hard to maintain sleep homeostasis across a shift work period. These findings may elucidate why work risk is cumulatively compromised across consecutive work shifts (Folkard & Tucker, 2003), as the balance between sleep and wakefulness becomes increasingly aberrant across a period of night work.

4.2.1 Can Sleep Predict Markers of Cortical Protein Synthesis?

Protein synthesis is regulated by both circadian factors (Lipton et al., 2015) and sleep (Grønli et al., 2014). Specifically how circadian rhythms and sleep parameters independently regulates protein synthesis, is however not well known.

As shown here and previously in the shift work model (Marti et al., 2017), rest work reduced the expression of the translational markers pS6K1 and pBMAL1 in the PFC relative to their time-matched controls. Sleep parameters did however not explain these observed

differences findings. A negative partial correlation between pS6K1 and NREM sleep was however found in the 24 hour data. Nevertheless, in the regression model, work was the major predictor across all steps. This supports previous findings of pS6K1 being unaffected in mice hippocampi after 5 hours of sleep deprivation (Tudor et al., 2016). Other studies have however found pS6K1 to be reduced in the mice hippocampi after 5 hours of sleep deprivation (Frolinger et al., 2018) and in the cerebellum and striatum following 10 hours of sleep deprivation (Kam et al., 2019). As discussed in Kam et al. (2019), conflicting findings of sleep regulating pS6K1 could be due to length of sleep deprivation. One could argue that the shift work model was even more impactful in depriving sleep, as our animals were in the wheels for 8 hours across 3 consecutive days and therefore sleep deprived already before tissue collection after W3. Nevertheless, none of the aforementioned findings did sample the PFC. It should therefore not be excluded that expression of pS6K1 could be differently regulated by sleep across brain regions. In sum, as pS6K1 only showed marginal associations sleep parameters, our results indicate that pS6K1 may be modulated by circadian factors in the PFC.

peIF4E was significantly reduced in rest workers relative to their controls. It was further shown that peIF4E strongly correlated positively with NREM episode lengths based on 2 hour data. This was further supported in the regression model, where the vast majority of the predictive power came from NREM sleep episode lengths. A specific influence of sleep on peIF4E expression has been demonstrated (Tudor et al., 2016). Seibt et al., (2012) moreover performed monocular deprivation and then inhibited mTORC1 signaling prior to 6 hours of sleep. This caused plasticity to be reduced in the primary visual cortex, which they argue represents that translation primarily occurs during sleep. Our results may contribute to these findings, by showing that having consolidated sleep is more influential on pS6K1 expression, relative to other NREM sleep parameters.

As an association with peIF4E and sleep was only found for the 2 hour data, it further suggests that expression of peIF4E is dependent on more recent sleep history, rather than sleep prior to work. Translation of eIF4E mRNA occurs very fast. Per codon in the mRNA, it takes approximately 50 milliseconds to gather the respective amino acids in eukaryotic cells (Prabhakar, Choi, Wang, Petrov & Puglisi, 2017) and an eIF4E protein consists of approximately 170 amino acids (Joshi, Cameron & Jagus, 2004). Due to the rapid rate of translation, it could therefore be that peIF4E expression is more reliably measured based on data closer to euthanization.

In sum, altered protein synthesis in a night work context can be ascribed to both circadian and sleep specific parameters. As it is shown that pBMAL1 and pS6K1 is predicted by type of shift work, it suggests that these proteins are altered due to circadian factors. As peIF4E is predicted by length of NREM sleep episodes, our data suggests that this translational protein is regulated by sleep.

4.2.2 BDNF predicted by Sleep Drive and Consolidated NREM sleep

Synaptic potentiation occurs during waking. To maintain synaptic homeostasis, SWA sleep is crucial for downscaling the synapses (Esser, et al., 2007; Tononi & Cirelli, 2006). One protein contributing to synaptic potentiation is BDNF (Lu, 2003; Taishi et al., 2001). The presented results predicted BDNF expression from SWA during QW in the PFC. These findings may cohere with studies showing a positive relationship between BDNF and SWA during NREM sleep in humans (Bachmann et al., 2012) and rodents (Faraguna et al., 2008; Huber et al., 2007). The similar parameter measured here however, SWA in NREM sleep, was not associated with BDNF. Nonetheless, the theory of synaptic homeostasis postulates that neuronal potentiation is reflected in SWA (Tononi & Cirelli, 2014). It may therefore be argued that intrusion of SWA in the awake brain during a period of rest work, could be

manifested in strengthened synaptic connections parallel with increased expression of BDNF. In humans (Huber et al., 2012) and rodents (Liu et al., 2010), neuronal excitability progressively increases across wakefulness and sleep deprivation. Our findings may therefore cohere with such literature, where BDNF may be one factor contributing to synaptic potentiation. As it was additionally shown that rest workers had a steeper incline in sleep drive across the work period, this might further indicate that increased BDNF expression is one underlying neurobiological marker of an increased sleep drive during wakefulness.

For BDNF and sleep, NREM sleep consolidation was associated with BDNF expression in the 24 hour data. As indicated here and previously in the shift work model (Grønli et al., 2017), the average duration of NREM sleep episodes are shorter between periods of rest work, relative to active work. The results might therefore indicate that particularly consolidated NREM sleep is important for downscaling of the synapse during sleep. The particular relationship between BDNF expression and NREM sleep consolidation is however not much explored. In mice, one study has manipulated sleep consolidation via stimulation of wake-promoting neurons, without affect quality or length of sleep. If stimulation was given after learning in the novel objects task, later performance was significantly reduced (Rolls et al., 2011). As BDNF has also been shown to predict performance in the novel object task (Huber et al., 2007), it is therefore not unlikely that our findings of sleep consolidation predicting BDNF could be a sleep parameter mediating cognitive performance, moreover synaptic potentiation.

It should also be noted that BDNF was not associated with the same sleep and wakefulness parameters in the 2 hour and 24 hour data. As the 2 hour data was a period shortly after the end of W3, it was therefore a period with high sleep pressure among the animals, which likely influenced the predictive power of SWA during QW on BDNF. That sleep pressure was high after shifts, may be supported by how both groups showed a steady

decline in SWA during NREM sleep after W2 (figure 14). As the animals were allowed to

sleep for 16 hours during the 24 hour data, it may be that sleep pressure was further attenuated

and thus reduced associations between BDNF and SWA in QW for the 24 hour data. On the

other hand, that as the 24 hour data involved more sleep data, it likely increased the predictive

power for NREM sleep episode lengths and BDNF. Thus, when examining continuous sleep

and wakefulness data in relation to protein expression, it might be crucial to define the correct

time frame to capture an effect.

In sum, our findings of SWA in QW predicting BDNF might indicate synaptic

potentiation. Moreover, disruption of consolidated sleep may particularly be an influential

factor in regulating BDNF expression.

4.2.3 Day shift or Night Shift, does it Matter When it Comes to BDNF?

It was an interesting finding that BDNF was practically unchanged after both rest

work and active work, relative to baseline control animals. Nor did work provide any

predictive power for BDNF in the regression model. This indicates that in a night work

context, predicting expression of BDNF is not reliably done based on whether one is working

night shifts or day shifts. This is however rather strange as it is here found group differences

in SWA during NREM sleep and length of NREM sleep episodes, both predicting BDNF

expression.

Speculatively, this could be reflected in individual susceptibilities to night work,

which is prominent in the human population. Furthermore, research on genes that influences

susceptibility is very limited (Saksvik, Bjorvatn, Helland, Sandal, Pallesen, 2011).

Specifically for BDNF, it has been shown that individuals with the naturally occurring

Val66Met polymorphism, show less time in and quality of SWS after prolonged waking,

relative to non-carriers of gene (Bachmann et al., 2012). This indicates that they may have a

heightened susceptibility to night work, as they show reduced restorative functions of sleep.

Whether such a genetic variance was prominent in our animals is however unknown.

Nevertheless, our animals were outbred, which is supposed to reflect the genetic

heterogeneity of the human population (Festing, 2010). Overall, genetic variations are often

prominent across and within different breeding colonies (Brekke, Steele & Mulley, 2018). For

the sprague-dawley strain specifically, genetic variation has been shown within the same

colonies of animal vendors, which has been demonstrated to significantly affect within-group

differences in behavioral tasks (Fitzpatrick et al., 2013). As no differences in BDNF

expression were seen among rest workers and active workers, our data may therefore suggest

that BDNF expression is susceptible to individual variation.

4.2.4 The Mysterious Arc Protein

It was hypothesized that Arc expression would be associated with REM sleep

parameters. This was based on previous studies showing that Arc is upregulated by ACh

(Soulé et al., 2012), and REM sleep being associated with cholinergic activity (Diekelmann &

Born, 2010; Van Dort et al., 2015). At the group level, rest work reduced Arc expression

relative to their time-matched control animals. This replicates a previous finding in the shift

work model (Marti et al., 2017) and indicates that expression of the Arc protein is reduced

during night work. Although some predictive power was given for Arc and REM sleep

consolidation, the model was not significant which indicates that it was not a good fit for

predicting the Arc protein. As no prediction was found for Arc and sleep, it does not mean

that the findings contradictory to the literature. The hypothesis was based on sleep deprivation

experiments, which directly manipulated the amount of sleep as an independent variable. The

experimental paradigm used here is per se not a sleep deprivation paradigm, as sleep was not

directly manipulated. The alterations in sleep patterns are rather a consequence of a re-

scheduled activity-inactivity pattern in the animals. It could therefore mean that the shift work model did not sufficiently alter REM sleep parameters to affect Arc expression.

On the other hand, as a group difference was identified for Arc, it could however mean that Arc is also modulated by circadian factors. Such an argument may be supported by PFC Arc expression being upregulated in undisturbed animals at ZT0 and downregulated at ZT12 (Marti et al., 2017). In addition to PFC Arc mRNA being upregulated at ZT1 and downregulated at ZT11 (Calabrese et al., 2011). Thus, as it was not found a relationship with Arc and sleep, it may be suggested that our identified reduced Arc expression in rest workers may be due to circadian regulation of Arc in the PFC.

4.2.5 Wakefulness Episodes Predicts NPAS4

Due to NPAS4 being a recently discovered protein, the hypothesis for this protein being regulated by REM sleep was rather uncertain. Surprisingly, NPAS4 correlated with waking episodes and not with any sleep variables. This was furthermore supported in the regression model, where number of waking episodes predicted NPAS4 expression. Relative to the time-matched control animals, rest workers also increased their expression, whereas active workers largely maintained their expression. This indicates that rest work specifically alters NPAS4 expression in the PFC.

Current literature of NPAS4 mRNA in relation to REM sleep deprivation has shown to upregulate NPAS4 mRNA cortically (Narwade et al., 2017) and regular sleep deprivation to downregulate (Orozco-Solis et al., 2017). This indicates that sleep or wakefulness specifically affects NPAS4 expression. Comparing our data with these results is however hard, as correlations between mRNA levels and proteins often low due to there being a substantial amount of specific post-transcriptional and post-translational processes (Vogel & Marcotte, 2012). It is also not known whether NPAS4 is expressed differently across brain regions.

In sum, our findings indicate that NPAS4 at the protein level is upregulated in the PFC by simulated night work, where wakefulness seems to upregulate its expression.

4.3 Animal Models of Shift Work, How Valid are They?

The underlying purpose of establishing animal models, is to ultimately draw inference about a desired human phenomenon, however, without studying human subjects. Using rodent models for studying human sleep disorders has been a valuable tool for elucidating their underlying mechanisms (Toth & Bhargava, 2013). This, as the underlying physiological and neurochemical processes of sleep and wakefulness are similar between humans and rodents (Revel et al., 2009).

Animal models of night work examining sleep and wakefulness is not a wide spread field of research. As it is here shown that several sleep and wakefulness parameters and protein expression in the PFC are replicated, the present data strengthens reliability of the shift work model in studying sleep. On the other hand, it may also raise questions about the reliability of other measurements. Of notice here, is the measurement of NREM- and REM sleep episode lengths. These measurements were not significant (NREM sleep episode length however had a large effect size between the groups, indicating an effect). This could be due to a fundamental difference between human and rat sleep. As rats are polyphasic sleepers (Simasko & Mukherjee, 2009) and humans are not, they have a lot more awakenings and initiation of sleep episodes. It may therefore be that measuring sleep consolidation in rodents is more statistically demanding than in humans. This is not to say sleep consolidation measurements are not transferable to a human phenomenon or that having a significant p-value should always be required. However, it should be taken notice that sleep consolidation measures in rodents may require a higher sample power than in humans.

When using animal models to study a human phenomenon, construct validity may be

at stake in animal models. Our shift work model is based on lighting conditions being kept

constant, whereas activity patterns are organized within different time frames of the 24 hour

recurrent lighting conditions. The animals were kept awake, as confirmed by the EEG and

EMG measurements during the work sessions. This is similar to what is required of shift

workers, as they have to be active at times when they would usually sleep. As the provided

sleep and wakefulness data share similarities with human data (as discussed in 4.1.1 – 4.1.3),

it strengthens construct validity of sleep and wakefulness measurements the model.

A potential aspect that may decrease construct validity in the model, is the situational

awareness for the animals. In a human setting, one would accept to perform night work and

one would also be informed about when the night shift is over. It should therefore not be

excluded that a lack of situational awareness in the rats, may trigger a higher stress response

relative to a human setting of night work. This is worth mentioning, as other behavioral

animal models rely on situations being unpredictable. Examples for this, is the forced swim

test (Can et al., 2012) or tail suspension test (Steru, Chermat, Thierry, & Simon, 1985), where

the measurements are based on animal admitting defeat.

As the shift work model allows for control of confounding variables, it is a major

strength of the model. One example of a confounding variable is routines prior going to sleep.

Such as reading from electronic devices that emit light, in where the light may delay the onset

of and overall SWA in subsequent sleep (Grønli et al., 2016). This is undoubtedly one of

many examples on how everyday environmental stimuli may affect sleep and wakefulness

patterns in naturalistic study of human shift work. Having full control of what the sample is

exposed to, therefore allows for a proper experimental setting to study the causal mechanisms

of how specifically shift work affects sleep and wakefulness parameters. Studying shift work

in experimental models is therefore highly valuable to determine causal relationships between

night work and sleep-wake parameters.

In sum, the it can therefore be argued that the presented results provide reliability to the shift work model, as sleep, wakefulness and protein data were replicated. As the data coheres with human studies of sleep and shift work, it further provides naturalistic validity to the model and may be a highly valuable tool to experimentally elucidate on the effects of shift work.

4.4 Limitations of the Study

A major limitation of this experiment was sample size. Grønli et al. (2017) collected sleep-wake data from 27 animals, whereas this experiment from 16. Sample sizes moreover varied across analyses, which may have caused varying statistical power. As a consequence, the likelihood of committing type I and type II errors increases, which further may reduce replicability (Button, et al., 2013), one of the overall aims of this experiment. Sleep, wakefulness and protein data were however replicated, indicating that the shift work model is reliable with even lower sample sizes. Nevertheless, a reduced sample size particularly affected the 2 hour sleep and wakefulness data, where a third of the active workers data were lost. This happened due to technical difficulties.

For the protein data, it is also worth addressing that tissue sampling was only done prior to work and after work. As addressed for peIF4E and BDNF, different predictions were made from different time spans of sleep and wakefulness data. As there were no baseline protein measurements 24 hours and 2 hours prior to euthanization, it cannot be said with certainty how protein data changed within these time spans. This would also be relevant for accurately addressing how rapidly the measured proteins are expressed in relation to sleep and wakefulness parameters.

Stepwise hierarchical regression are subjective analyses. Involved predictors are selected based on previous literature and the researcher's hypotheses. Thus, there is a

possibility that some appropriate sleep and wakefulness parameters were not included in the analysis. To reduce this, it was therefore run partial correlations between sleep and wakefulness parameters from the initial analyses that were followed up with regression analyses.

4.5 Future Perspectives

In the experimental conditions of this study, a constant rest work condition was compared to a constant day work condition. Night work schedules may be more complex than this. One example is rotating shifts, where an individual may alter between working morning, evening and night shifts (Åkerstedt, 2003). It will additionally be possible to rapidly adjust the experimental design, to determine which work schedules that may be more appropriate than others. Rats also mature more quickly than humans. For rats, 30 years is about the equivalent of one human year (Andreollo, Santos, Araújo & Lopes, 2012). The rodent shift work model may therefore be a valuable tool to effectively elucidate the long term effects of different work schedules.

There are several different methodologies that would elucidate on the findings of this thesis. For protein markers, it would be further interesting to see how *in vivo* neuronal firings would be predicted. This could be possible by measuring local field potentials and would elucidate very accurately on how neuronal functioning is affected by night work. This would especially enlighten on the associations between synaptic potentiation and night work. Moreover, behavioral tests would more explicitly indicate how cognitive functions are altered during shifts. Indications of impaired spatial memory has recently been demonstrated in the shift work model (Marti et al., 2019), but it would further be interesting to see how more complex PFC dependent tasks are altered.

5.0 Conclusion

This thesis demonstrates that previous findings of sleep and wakefulness changes after rodent simulated shift work are replicable. This, together with replicable data on markers of translation and synaptic plasticity in the PFC, adds to the reliability of the animal model of shift work. Construct validity is also strengthened, as it can be drawn parallels between collected sleep and wakefulness data and human data. The thesis shows that type of work, sleep and wakefulness predicts different aspects of cortical translational activity and synaptic plasticity. These findings provide indications that changes in sleep and wakefulness during a night shift period, may contribute to impaired cognitive functioning, and moreover compromised work risk during night shifts.

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Appendix A

Approval from Norwegian Food Safety Authority

UiB - BBB, Bygg for biologiske basisfag
Jonas lies vei 91
5009 Bergen

Att. Janne Grønli

Fakturaref:	Org. Nr 874789542
Vår ref:	2016/263080
Dato:	08.10.2018
Org.nr:	874789542

Statens tilsyn for planter, fisk, dyr og næringsmidler

Mattilsynet

VEDTAK OM BRUK AV FORSØKSDYR - FOTS ID 11321
Virksomhetsnummer 004: UiB - BBB, Bygg for biologiske basisfag

Behandlet av Mattilsynet, 08.10.2018.

Saken gjelder
Dette vedtaket gjelder melding om endringer i godkjent forsøk med FOTS id 11321. Meldingen er behandlet som en søknad, grunnet mulighet for økt samlet belastning ved bruk av atferdstesten Morris water maze. Det er tidligere gitt tillatelse til bruk av 924 rotter i forsøk. Formålet med forsøket er å øke forståelsen av sammenheng mellom ikke-restorativ søvn og kognitiv svekkelse hos nattarbeidere. Dyrene får implantert en trådløs sender med avledninger som registrerer søvn/våkenhet, kroppstemperatur og hjerterate. Dyrene deles i hvert forsøk i 3 grupper; dagarbeidere, nattarbeidere og kontroller. For enkelte grupper vil det utføres atferdstester og kognitive tester etter endte skiftarbeidsperioder.

Endringene det søkes om består i å endre rottestamme, legge til to nye medarbeidere og bruke Morris water maze for å teste kognitiv fleksibilitet.

Forsøket er klassifisert som moderat belastende.

Dokumenter i saken:

1. Søknad om endring, FOTS id 11321 datert 03.05.2018.
2. Tilleggsinformasjon i e-post fra Anne Marie Rød datert 03.10.2018.
3. Godkjenningsvedtak datert 01.02.2017.

Vedtak
Mattilsynet gir Janne Grønli tillatelse til å gjennomføre de omsøkte endringene i forsøket. Forsøket skal utføres ved UiB, BBB, i perioden 08.10.2018 – 31.01.2021.
Vedtaket er fattet med hjemmel i forskrift 18. juni 2015 nr. 781 om forsøk med dyr (forsøksdyrforskriften) §37, jf. § 6.

Begrunnelse
Vi vurderer at formålet med endringen av forsøket og bruken av dyr er tilfredsstillende beskrevet i søknaden og tilleggsinformasjonen, slik at kravene i forsøksdyrforskriften § 10 (formål med forsøk), § 11 (metoder, teststrategier og endepunkter) og § 9 (erstatning, reduksjon og forbedring) er oppfylt. På bakgrunn av en vurdering av hvor nyttig forsøket er i forhold til belastningen for dyrene mener vi at dyr ikke utsettes for unødvendige belastninger, jf. forsøksdyrforskriften § 1.

Forsøket er klassifisert som moderat belastende, jf. forsøksdyrforskriften vedlegg B.

Vi forutsetter at alle som deltar i forsøket har fått tilstrekkelig utdanning og opplæring og at deres kompetanse vedlikeholdes, jf. forsøksdyrforskriften § 24.

Godkjenningen forutsetter at ansvarlig søker er innforstått med ansvaret til person med særskilt kontrollansvar (PMSK) og dyrevelferdsenheten når det gjelder å følge opp forsøkenes påvirkning på dyrene. Ved uenighet om humane endepunkter og eventuell stans av et forsøk

www.mattilsynet.no

| **Mattilsynet**
Avdeling for nasjonale oppgaver | Saksbehandler: Vera Klafstad Rodas
Tlf.: 22 40 00 00
E-post: postmottak@mattilsynet.no | Postadresse:
Felles postmottak, Postboks 383
2381 Brumunddal |

skal PMSK og/eller dyrevelferdsenheten avgjøre og treffe beslutning, jf. forsøksdyrforskriften §§ 25 og 26.

Ved behov for endringer av den godkjente søknaden må dette sendes til Mattilsynets forsøksdyrenhet via FOTS som søknad/ melding om endring.

Vi minner om kravet om årsrapportering, jf. forsøksdyrforskriften § 36. Manglende rapportering vil kunne medføre at vi trekker tilbake denne godkjenningen og avslår nye søknader.

Vi innkrever et gebyr på 1535 NOK (+ 100 NOK i administrasjonsgebyr) for behandling av søknad om godkjenning av dyreforsøk, jf. forskrift 13. februar 2004 nr. 406 om betaling av gebyrer for særskilte ytelser fra Mattilsynet jf. § 5.

Vedtak kan påklages til Mattilsynet, jfr. lov 10 feb 1967 om behandlingsmåten i forvaltningssaker(forvaltningsloven) § 28. Klagefristen er 3 uker fra mottak av dette brev, jfr.forvaltningsloven § 29. Klagen stiles til Mattilsynet, Hovedkontoret, men sendes via avdeling for nasjonale oppgaver.

Med hilsen

Ole Aamodt
avdelingssjef

Med hilsen

Vera Klafstad Rodas
saksbehandler

Kopi:
personell med særskilt kontrollansvar
postmottak@mattilsynet.no

Appendix B

Table 1

Main group effects and interaction effects from rANOVA analyses of minutes in quiet-,

intermediate- and active wakefulness during the 3 work sessions. Baseline corresponds to

work hours in groups

	Group			Group x Time interaction		
	Df	*F*	*p*	*Df*	*F*	*p*
Quiet Wakefulness						
Baseline	1, 12	15.92	.002	3, 36	2.91	.048
W1	1, 12	0.41	.536	3, 36	0.41	.536
W2	1, 12	0.15	.701	3, 36	0.15	.701
W3	1, 12	2.28	.157	3, 36	0.58	.632
Intermediate Wakefulness						
Baseline	1, 13	617.71	<.001	3, 36	3.78	.018
W1	1, 13	0.00	.949	3, 36	0.56	.643
W2	1, 13	3.27	.094	3, 36	3.79	.018
W3	1, 13	0.11	.741	3, 36	0.09	.963
Active Wakefulness						
Baseline	1, 13	146.99	<.001	3, 36	2.10	.116
W1	1, 13	0.27	.611	3, 36	0.87	.465
W2	1, 13	0.83	.377	3, 36	2.13	.113
W3	1, 13	1.32	.272	3, 36	2.64	.063

W = Work, Df = Degrees of Freedom, p = significance value.

Table 2

Main group and interaction effects of wakefulness and sleep in the 16 hours between shifts.

Baseline corresponds to the same hours

	Group			Group x Time interaction		
	Df	*F*	*p*	*Df*	*F*	*p*
Quiet Wakefulness						
Baseline	1, 13	8.29	.012	7, 91	5.00	<.001
W1	1, 13	.170	.687	7, 91	6.73	<.000
W2	1, 13	1.374	.262	7, 91	3.59	.002
Intermediate Wakefulness						
Baseline	1, 13	124.14	<.000	7, 91	32.00	<.001
W1	1, 13	.920	.354	7, 91	7.00	<.001
W2	1, 13	11.268	.005	7, 91	2.89	<.001
Active Wakefulness						
Baseline	1, 13	63.33	<.000	7, 91	16.01	<.001
W1	1, 13	.165	.690	7, 91	2.21	.041
W2	1, 13	1.932	.187	7, 91	2.02	.061
NREM Sleep						
Baseline	1, 13	52.54	<.000	7, 91	27.03	<.001
W1	1, 13	1.173	.210	7, 91	7.32	<.001
W*2*	1, 13	4.012	.066	7, 91	4.53	<.001
REM Sleep						
Baseline	1, 13	116.65	<.000	7, 91	25.50	<.001
W1	1, 13	.974	.341	7, 91	3.45	.003
W2	1, 13	1.437	.251	7, 91	1.35	.236

W = Work, Df = Degrees of Freedom, p = significance value.

Table 3

Total minutes spent in wakefulness and sleep states 2 hours prior to euthanization.

Stage (in minutes)	AW		RW	
	M	*SEM*	*M*	*SEM*
Quiet Wake	30.42	0.69	20.74	0.88
Intermediate wake	8.54	0.46	5.89	0.80
Active Wake	15.20	0.36	9.17	0.44
NREM Sleep	60.67	5.11	72.04	6.38
REM Sleep	1.67	.96	11.57	1.98

Note: AW = active work, RW = rest work, M = mean, SEM = standard error of mean.
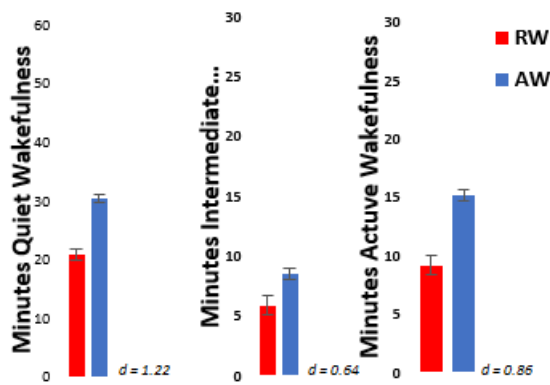
Figure 1. States of wakefulness in the two hours before euthanization. d = cohens` d.
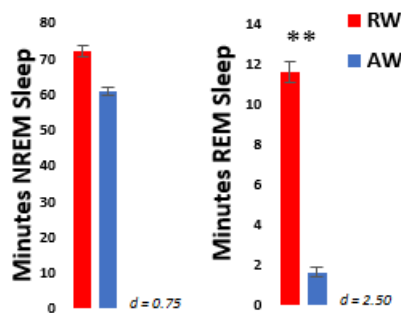
Figure 2. States of sleep in the two hours before euthanization. d = cohens` d. ** p<.01.

a)

	pS6K1	pBMAL1	peIF4E	BDNF	Arc	NPAS4
Total Minutes NREM Sleep	0,19	0,34	0,13	-0,38	0,01	0,44
Total Minutes REM Sleep	-0,04	-0,03	0,21	-0,13	0,12	0,37
NREM Sleep episode Length	0,07	0,34	0,91**	-0,09	0,02	0,32
REM Sleep episode Length	-0,21	-0,24	-0,07	-0,13	0,14	0,31
Wake episode Length	0,12	0,22	0,61	0,07	-0,11	0,00
NREM Sleep episode Amount	-0,03	-0,03	-0,41	-0,30	0,23	-0,35
REM Sleep episode Amount	-0,09	0,28	-0,07	-0,03	0,12	0,19
Wake Bout episode Amount	0,41	-0,21	0,20	-0,28	0,28	-0,56
SWA in Quiet Wake	-0,53	-0,18	0,38	0,73*	0,61	-0,26
SWA in NREM Sleep	-0,06	-0,04	0,10	-0,40	0,05	0,15
Gamma in Quiet Wake	-0,26	-0,29	-0,55	0,61	0,32	-0,31
Gamma in REM Sleep	0,11	-0,25	-0,04	-0,21	0,09	0,10

b)

	pS6K1	pBMAL1	peIF4E	BDNF	Arc	NPAS4
Total Minutes NREM Sleep	-0,58 *	0,22	0,00	0,47	0,00	0,37
Total Minutes REM Sleep	-0,09	0,13	0,33	-0,15	0,30	0,00
NREM Sleep episode Length	-0,48	0,27	0,17	0,90 ***	0,48	0,24
REM Sleep episode Length	-0,16	-0,40	-0,18	-0,20	-0,08	-0,38
Wake episode Length	0,25	0,36	-0,40	0,28	0,26	0,38
NREM Sleep episode Amount	0,30	-0,02	-0,22	-0,82 ***	-0,54	-0,25
REM Sleep episode Amount	0,02	0,24	0,27	-0,04	0,40	0,08
Wake episode Amount	0,20	-0,49	0,14	-0,46	0,18	-0,53 *
SWA in Quiet Wake	0,07	-0,28	0,45	0,60 *	0,49	0,03
SWA in NREM Sleep	-0,09	0,36	-0,38	0,44	0,49	0,31
Gamma in Quiet Wake	-0,35	-0,42	0,57	0,27	0,01	0,19
Gamma in REM Sleep	0,01	-0,42	-0,04	-0,43	-0,27	-0,12

Figure 2 a & b. Partial correlations between sleep-wake parameters and protein markers of translational activity and plasticity. (a) Shows sleep-wake data 2h prior to euthanization. (b) 16 hours after W2 and during W3 (24 hours). Shown values are r-values. Significant values denoted at *p<.05, ** p<.01 and ***p<.000. Scatterplots are shown for significant values

Table 4

P-values and n from partial correlation of 2h data prior to euthanization.

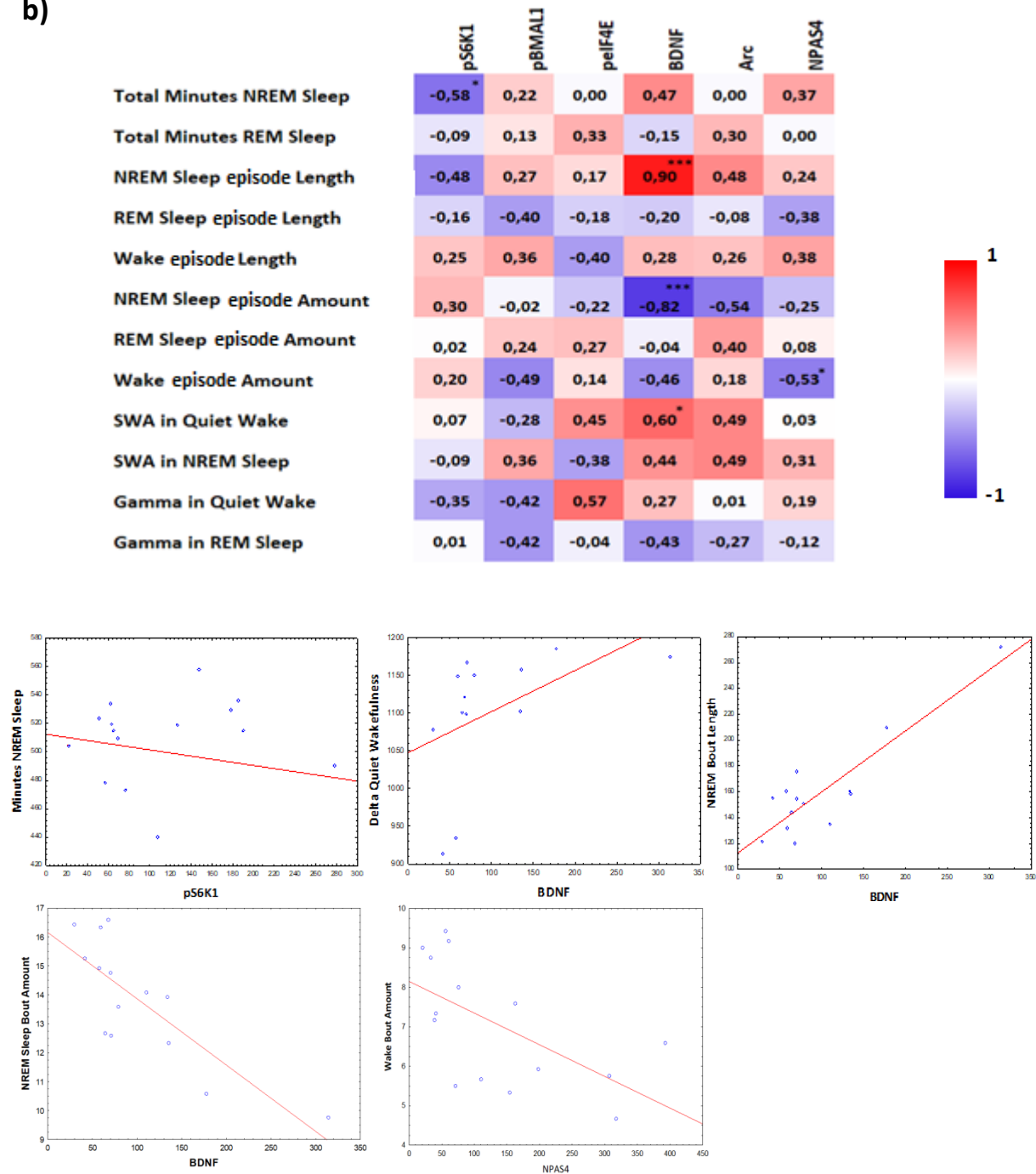|	Min. NREM Sleep	Min. REM Sleep	NREM Sleep Bouts length	REM Sleep Bout length	Wake Bout Length	NREM Sleep Bout Amount	REM Sleep Bout Amount	Wake Bout Amount	SWA in QW.	SWA in NREM Sleep	Gamma in QW	Gamma in REM Sleep
pS6K1												
p	.767	.604	.818	.513	.712	.924	.773	.188	.096	.642	.437	.978
n	13	13	13	13	13	13	13	13	12	12	12	12
pBMAL1												
p	.293	.809	.279	.457	.496	.385	.385	.508	.597	.935	.382	.914
n	13	13	13	13	13	13	13	13	12	12	12	12
peIF4E												
p	.764	.545	.002**	.864	.110	.319	.874	.639	.407	.507	.202	.547
n	9	9	9	9	9	9	9	9	8	8	8	8
BDNF												
p	.180	.241	.802	.709	.845	.362	.933	.410	.017*	.157	.062	.347
n	12	12	12	12	12	12	12	12	11	11	11	11
Arc												
p	.975	.823	.962	.720	.742	.544	.762	.465	.111	.843	.435	.917
n	10	10	10	10	10	10	10	10	9	9	9	9
NPAS4												
p	.182	.487	.310	.332	.978	.268	.547	.059	.441	.492	.353	.488
n	13	13	13	13	13	13	13	13	12	12	12	12

*Note: p = significance value, n = group sample size. *p<.05, ** p<.01 and *** p<.000*

Table 5

P-values and n from partial correlation of 16 after W2 and W3 (24 hour) data

	Min NREM Sleep	Min REM Sleep	NREM Sleep Bout Length	REM Sleep Bout Length	Wake Bout Length	NREM Sleep Bout Amount	REM Sleep Bout Amount	Wake Bout Amou	SWA in QW.	SWA in NREM Sleep	Gamma in QW	Gamma in REM Sleep
pS6K1												
p	.037*	.765	.096	.611	.501	.297	.949	.486	.832	.770	.245	.970
n	15	15	15	15	15	15	15	15	14	14	14	14
pBMAL1												
p	.454	.667	.376	.174	.202	.532	.425	.072	.358	.238	.153	.151
n	15	15	15	15	15	15	15	15	14	14	14	14
peIF4E												
p	.993	.384	.671	.646	.256	.538	.442	.709	.227	.313	.112	.912
n	11	11	11	11	11	11	11	11	10	10	10	10
BDNF												
p	.124	.632	.000***	.525	.361	.001***	.897	.110	.038*	.152	.399	.163
n	14	14	14	14	14	14	14	14	13	13	13	13
Arc												
p	.999	.401	.165	.829	.446	.086	.224	.601	.149	.151	.971	.459
n	12	12	12	12	12	12	12	12	11	11	11	11
NPAS4												
p	.212	.990	.427	.201	.186	.381	.795	.049*	.927	.301	.533	.692
n	15	15	15	15	15	15	15	15	14	14	14	14

Note: p = significance value, n = group sample size. *p<.05, ** p<.01 and *** p<.001