Effects of early life stress and chronic mild stress exposure in adulthood on sleep and wakefulness in rats

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Abstract

The postnatal period is important for normal brain development across species, and environmental manipulations during this period have been shown to have significant impact on sleep later in life. The main aim of this study was to investigate the effect of early life stress and chronic mild stress (CMS) in adulthood on sleep and wakefulness, and further to investigate how sleep and wakefulness were affected by different individual mild stressors. Male, newborn rats (Wistar rats, n = 13) were exposed to brief-maternal separation (BMS, 10 min daily) and long-maternal separation (LMS, 3 h daily) during postnatal day 2-14. In adulthood, all animals were exposed to 4 weeks of (CMS). Electroencephalography (EEG) and electromyography (EMG) were recorded continuously during 24 h baseline and one week during the CMS protocol to assess sleep and wakefulness. Results showed no overall effect of early life stress on sleep and wakefulness in either active phase or in inactive phase, as LMS and BMS offspring displayed similar time in wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep. CMS exposure affected sleep and wakefulness in both LMS and BMS offspring. One week of CMS exposure affected sleep and wakefulness similarly in LMS and BMS offspring. Across all CMS days during active phase, wakefulness was reduced and SWS, REM sleep and total sleep time were increased, whereas during inactive phase, wakefulness was increased and SWS, REM sleep and total sleep time were reduced. Stressors "food deprivation" and "social stress" induced a stronger effect in LMS compared to BMS offspring. During food deprivation LMS offspring showed less wakefulness and more total sleep time compared to BMS offspring. After exposure to social stress the LMS offspring spent more time in wakefulness, less time in SWS and had less total sleep time compared to

BMS offspring. Overall, these findings suggest a difference in stress reactivity in LMS and BMS offspring and add to the literature on the consequences of early life stress in combination with chronic mild stress in adulthood on sleep and wakefulness, both during active and inactive phase.

Sammendrag

Den postnatale perioden har på tvers av arter vist seg å være viktig for normal utvikling av hjernen, og miljømessige påvirkninger i løpet av denne perioden har vist å påvirke søvn senere i livet. Hovedmålet med denne studien var å undersøke effekten av tidlig livsstress og kronisk mildt stress i voksenlivet på søvn og våkenhet, videre var målet å undersøke hvordan søvn og våkenhet ble påvirket av ulike milde stressorer. Nyfødte hannrotter (Wistar rotter, n = 13) ble eksponert for kortvarig separasjon fra mor (BMS, 10 min daglig) eller langvarig separasjon fra mor (LMS, 3 timer daglig) i løpet av postnatal dag 2-14. Som voksne ble de samme dyrene eksponert for 4 uker med kronisk mildt stress. For å måle søvn og våkenhet ble elektroencefalografi (EEG) og elektromyografi (EMG) registrert kontinuerlig i løpet av 24 t baseline og i en uke under eksponering for kronisk mildt stress (CMS). Resultatene viste at det ikke var en overordnet effekt av tidlig livsstress på søvn eller våkenhet i hverken aktiv fase eller inaktiv fase, da både LMS og BMS avkom hadde like mye våkenhet, SWS og REM søvn. Eksponering for CMS påvirket søvn og våkenhet i både LMS og BMS avkom. En uke med CMS eksponering påvirket søvn og våkenhet likt i LMS og BMS avkom. På tvers av alle CMS-dagene var våkenhet redusert, mens SWS, REM søvn og total søvntid var økt i løpet av aktiv fase. For inaktiv fase var våkenhet økt, mens SWS, REM søvn og total søvntid var redusert. Stressorene «matdeprivasjon» og «sosialt stress» induserte en sterkere effekt i LMS avkom sammenliknet med BMS avkom. Under matdeprivasjon viste LMS avkom mindre våkenhet og mer total søvntid sammenliknet med BMS avkom. Etter eksponering for sosialt stress viste LMS avkom mer våkenhet, mindre SWS og redusert total søvntid sammenliknet med BMS avkom. Resultatene viser at det er en forskjell i stressreaktivitet hos LMS og BMS

avkom, og bidrar med nye funn hva gjelder konsekvenser av tidlig livsstress i kombinasjon med kronisk mildt stress i voksenlivet på søvn og våkenhet, både for aktiv og inaktiv fase.

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To write this thesis during a global pandemic has been both challenging, and frustrating, but I reached the finish line - eventually.

Lastly, I would like to thank everyone at the Department of Biological and Medical Psychology for an academic environment beyond great.

My contribution to the project

During my master project I was a part of Bergen Stress and Sleep group (BSSG) at Department of Biological and Medical Psychology, University of Bergen. This project is a part of the larger ongoing project "The early life condition – A translational study of affective and behavioural outcomes and genetic modulation", and data have been collected in 2009.

Previous studies from the project found differences in sleep and wakefulness between rat offspring that had been exposed to either long-maternal separation (LMS) or brief-maternal separation (BMS) during early life and after exposure to chronic mild stress (CMS) in adulthood (Mrdalj et al., 2013); and found different thermoregulatory responses to specific mild stressors (Mrdalj et al., 2014). These studies did not investigate sleep and wakefulness during the animals' active phase, and they did not investigate sleep and wakefulness *during* the exposure to chronic mild stress. The aim for my project was therefore first to investigate sleep and wakefulness in LMS and BMS offspring during both active and inactive phase. Further aim was to investigate how sleep and wakefulness were affected *during* CMS exposure, and more specifically; how different mild stressors affected sleep and wakefulness in both LMS and BMS offspring.

To achieve this, I have analysed electroencephalographic (EEG) and electromyographic (EMG) data recorded in LMS and BMS animals (n = 13). I was first trained in rat sleep scoring by my supervisor. Between November 2019 and April 2020, I manually scored the data in 10 second epochs using the sleep analysis tool "Neuroscore". Each animal had 1 x 24 h baseline recording and 7 x 24 h recording during CMS. In total,

there was 898 560 epochs of 10 s (equivalent to 2496 hours of EEG data). Each epoch was manually checked and re-scored if needed, to ensure the quality of the scoring. Furter, I used the analysis tool SLEEP-report app in MATLAB (developed by Professor Jonathan Wisor at Washington State University) to extract detailed data about sleep and wakefulness, for each animal. The data were further processed in Excel to prepare for the statistical analyses. I also performed all statistical analyses under supervision.

This is the first study investigating sleep and wakefulness in rats with a history of postnatal maternal separation in combination with exposure to chronic mild stress in adulthood during the rats' active phase. My research and findings will therefore contribute to extended knowledge in the field of early life stress and its effects on sleep and wakefulness in adulthood.

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Abbreviations

ANOVA	analysis of variance
AASM	American Academy of Sleep Medicine
BMS	brief maternal separation
CMS	chronic mild stress
EEG	electroencephalography
EMG	electromyography
EOG	electrooculography
Hz	hertz
h	hour
HPA	hypothalamus-pituitary-adrenal
LMS	long maternal separation
MS	maternal separation
NH	non-handling
NREM	non-rapid eye movement
PND	postnatal day
PSG	polysomnography
REM	rapid eye movement
SWA	slow wave activity
SWS	slow wave sleep

1. Introduction

During early life, the mother-infant interaction is important for the development of the offspring. Exposure to early life stress may influence the developing brain in lasting ways (McEwen, 2011), and stressful events early in life have been linked to a heightened risk for psychopathology in adulthood. One suggested explanation has been increased sensitivity to stress (Heim & Nemeroff, 2002; Lupien et al., 2009). Clinical studies also indicate that early life stress can induce long-term negative outcomes on sleep in adulthood (Bader et al., 2007; Schäfer & Bader, 2013).

Maternal separation is a widely used animal model to induce early life stress in offspring depending on the duration of the separation. It has been found that separation of rat offspring from the mother in the first weeks of life can have an effect on brain activity, increase anxiety- and depression-like behaviour and increase stress reactivity in adulthood (Mrdalj et al., 2013; Plotsky & Meaney, 1993). There are several studies on animals focusing on anxiety-like and depression-like behaviour as a consequence of early life stress, but few studies have investigated the consequences of early life stress on sleep later in life. Findings from the studies are inconclusive as both increased total sleep time, increased number of awakenings, and prolonged or reduced time in rapid eye movement REM sleep have been reported (Feng et al., 2012; Sampath et al., 2014; Tiba et al., 2004). Even fewer studies have investigated how the combination of early life stress and stress later in life affect sleep (Mrdalj et al., 2013; Tiba et al., 2004)

The following introduction will first focus on early development and the importance of maternal care, and then give an overview of early life stress and animal models of early life stress. The first part of the introduction will also look into general findings on consequences

of early and later life stress. The second part of the introduction will then first describe sleep and sleep regulation, and then give an overview on the effects of early life stress on sleep and effect of chronic stress in adulthood on sleep. Lastly, an overview of the few studies looking at combination effect of early and later life stress on sleep in adulthood will be given.

1.1 Early development and early life stress

1.1.1 Importance of maternal care

Brain development starts during early prenatal life (before birth) and continues during postnatal life (after birth), childhood and adolescence through both programmed (genes) and experience-dependent events (Tierney & Nelson, 2009). In most mammalian species, active maternal care and external stimuli, especially mother-infant relationship, are some of the most important preconditions for the survival of the offspring. The quality and frequency of maternal care has been shown to affect the maturation of brain, cognition, and emotion, as well as the behaviour of the offspring (Curley & Champagne, 2016; Rutter & Rutter, 1993).

In humans the importance of positive mother-infant attachment has been addressed several times in the field of psychology, evolving from the pioneering work from primarily Ainsworth and Bowlby. Attachment, according to Ainsworth (1963) is a "secure base from which to explore". This base is the foundation for further healthy development (Bowlby, 1969; Bowlby, 1982). Securely attached infants tend to have more favourable long-term outcomes with respect to cognitive, social and behavioural domains, while insecurely attached infants are more likely to have adverse outcomes (Flaherty & Sadler, 2011). Longitudinal studies in humans have documented that poor maternal care is a well-established risk factor

for neuropsychiatric diseases and psychopathology in adulthood (Sacks et al., 2017; Shin et al., 2013).

In rodents, active maternal care refers to behaviour such as licking and grooming and arched back nursing, to nourish and protect the litter during the first weeks of their development (Orso et al., 2019). During the first PNDs the rat mother spends 85% of her time feeding and nursing the offspring, with short periods away to provide food (e.g. 20-30 min). When the offspring become more mature, these periods away gradually increase (Grota & Ader, 1969). Ultrasonic vocalisation from offspring triggers active maternal care which promotes infant growth and maintains infant homeostasis via the regulation of physiology and behaviour. This maternal behaviour also regulates the development of the hormonal response system to stress in the offspring (Meaney, 2001).

During early PNDs in rodents, stress regulating mechanisms are immature as hypothalamus-pituitary-adrenal (HPA) axis undergoes maturational changes (van Bodegom et al., 2017). The HPA axis is an important hormonal response system which ensures that the body can respond to stressful events through the release of glucocorticoids (corticosterone in rodents, cortisol in primates) from the adrenal gland and return to normal state (homeostasis) as rapidly as possible through negative feedback mechanisms. In rodents, the period between PND 2-14 is termed the stress hyporesponsive period as the levels of corticosterone remain relatively low even if the offspring is presented to stimuli which normally elicit an increase of corticosterone in adult rats (e.g handling, exposure to novelty) (Levine, 1994). Moreover it has been shown that high levels of maternal licking and grooming strengthen the negative feedback mechanism of the HPA axis (Liu et al., 1997). Lack of active maternal care in early

life represents early life stress and can affect brain development and behaviour in the offspring.

1.1.2 Early life stress

The term early life stress includes both prenatal stress and postnatal stress. Through rest of the thesis the term "early life stress" will refer to postnatal stress unless otherwise specified. Early life stress in humans refers to any event that exposes a child to physical and/or psychological stimuli that exceeds their capacity to successfully cope (Gunnar & Quevedo, 2007). Neglect, emotional and physical abuse, caregivers with psychiatric disorders, abandonment, parental loss, lack of primary care and deprivation of food or adequate shelter are examples of adverse events that can occur during childhood and adolescence (Bernstein et al., 2003).

A recent report from World Health Organization (WHO, 2020) reveals that adverse events during childhood and adolescence are highly prevalent. Nearly 3 in 4 children aged 2-4 years regularly suffer physical punishment and/or psychological violence from parents and caregivers. According to a national survey in Norway regarding violence and traumatic stress experienced by adolescents, 20% of the youth in the age group 12-16 years report that they have been exposed to psychological violence from caregivers (Hafstad & Augusti, NKVTS, 2019). This number is at the same level as in other Nordic countries (Jernbro & Janson, 2017). About 1 in 20 adults report that they have experienced childhood abuse in form of serious violence (causing visible marks), or that they have experienced violence frequently during their adolescence. One in 5 young people have experienced less serious violence such as hair tugging, pinching or being beaten with a flat hand (Stefansen & Mossige, 2016). Sexual abuse

has also been reported in 1 in 5 girls, and 1 in 14 boys before the age of 18 (Thoresen & Hjemdal, 2014; Stefansen & Mossige, 2016; NKVTS, 2018).

Orphanage rearing is another example of early life stress in humans since it lies outside of the bounds of a typical caregiving environment. About eight million children live in orphanages worldwide, and according to UNICEF there were roughly 140 million orphans worldwide in 2015. Little is known about the long-term effects of orphanage rearing. A study from 2019 showed that children who were exposed to institutionalized care as infants later in life showed blunted cortisol reactivity to psychosocial stressors, even years after being adopted into well-resourced, supportive families (Gunnar et al., 2019). Moreover, altered cortisol response may be a vulnerability marker for psychiatric disorders in adulthood (Bosch et al., 2012).

To provide insight in the underlying mechanisms and behavioural outcomes later in life following early life stress, animal models of early life stress have been extensively used. Studies on animals enable knowledge about the neurobiological alterations induced by early life stress and allow for controlled experimental manipulations that would be unethical to perform in humans (Zimmerberg & Shartrand, 1992). In animal models we can control the start, duration and intensity of the stress and type of stressor, allowing us to make comparisons. Conducting longitudinal studies in humans entails very high costs in terms of resources, time and expense compared with animal models, and depend on the subject' s memory of events that occurred in childhood. Hence, false memories are very common, especially when related to emotional memories. This also emphasizes the importance of developing good animal models of early life stress (Banqueri et al., 2017).

1.1.3 Animal models of early life stress

Animal models of early life stress were first used in the laboratory more than 50 years ago (Levine, 1957) and have since been extensively utilized to examine the long-term impact of early life stress on psychobiology and behaviour. Animal models of early life stress typically rely on manipulating maternal presence and care, because these are the major sources of early life stress experienced in humans.

The pioneering studies from Harlow and colleagues in the 1950's investigating the mother-infant bond in rhesus macaques' monkeys emphasized the importance of mother-infant interaction for normal cognitive and emotional development (Harlow et al., 1966). Infant monkeys were separated from their mothers and were given a "surrogate mother" consisting of either a simple construction of wire and wood, or a wire and wood construction covered in foam rubber and soft terry cloth. The monkey infants were then assigned to one of two conditions; in the first condition, the "wire-mother" had a bottle of milk, while the "cloth-mother" did not. In the second condition, the "cloth-mother" had a bottle of milk, while the "wire-mother" did not. Results showed that for both conditions, the monkey infants spent significantly more time with the "cloth-mother", compared to the "wire-mother", even when the "wire-mother" was the one offering the milk bottle. It is believed that the soft material the "cloth-mother" consisted of, simulated the comfort normally provided by the mother. This is one of the most famous studies in behavioural science, as it demonstrated the importance of maternal care and attachment between mother-infant (Harlow et al., 1966). This captured the interests of researchers, and during the following years, the relationship between maternal behaviour and offspring physiology, as well as manipulation of the mother-infant bond in rodents were investigated (Stern et al., 1973; Thoman & Levine, 1970).

One of the most used manipulations to produce a rodent (rats and mice) model of early life stress has been maternal separation. As rodent offspring are dependent on their mother for nutrition, thermoregulation, and protection, maternal separation therefore represents a stressor (Hofer, 1973; Kaffman & Meaney, 2007). In this paradigm, the offspring are daily exposed to maternal separation during the first two postnatal weeks of life (the stress hyporesponsive period). The duration of the separation is used to simulate different early life conditions (see *Table 1)*.

Brief-maternal separation (BMS) is a condition where offspring are exposed to repeated short separations from the mother for 10-15 min per day (see *Table 1)*. This simulates naturalistic conditions were the rat mother leaves the nest regularly for short periods of about ten minutes, but not longer than 1 hour, depending on the age of the offspring (Grota & Ader, 1969). BMS have been shown to increase active maternal care and reduce stress reactivity in the offspring later in life (Plotsky & Meaney, 1993). Brief-maternal separation early in life may therefore provide a "toughening up" effect as the offspring shows reduced responsiveness to stressors in adulthood (Mrdalj et al., 2014).

Long-maternal separation (LMS) is a condition where offspring are exposed to longer periods of separation from the mother than considered as natural, 3-6 h per day, simulating neglect of the offspring (see *Table 1*). This results in lack of active maternal care (tactile stimulation and licking and grooming) for a long period of time, which in turn affect the neurobiological development in the offspring. LMS is associated with anxiety-like behaviour in adulthood and hyper-reactivity in the HPA axis (increased excretion of stress hormones) upon exposure to stressors in adulthood (Daniels et al., 2004; Plotsky & Meaney, 1993). It has also been shown that prolonged separation alters maternal care upon reunion, and these

differences may be as important, if not more, than the lack of contact with the mother (Boccia & Pedersen, 2001).

LMS is usually compared to early life conditions involving brief-maternal separation (BMS) and/or rearing in the constant presence of the mother without separation and without handling (non-handling, NH), or to an animal facility housing and handling condition (animal facility rearing/AFR), where the mother and offspring are not separated, but they are exposed to some physical handling due to cleaning of the cage (Nylander & Roman, 2013) (see *Table 1*).

Non-handling (NH) describes the experimental condition where the mother and offspring are left undisturbed in the cage, with no manipulation or handling of the animals. Unlike the AFR condition, there is no experimenter contact, and no cleaning of the cages. However, previous studies have shown that AFR and NH offspring can show a phenotype similar to offspring exposed to LMS or maternal deprivation (Mourlon et al., 2010; Pryce & Feldon, 2003). This may be caused by a change in natural maternal behaviour as the mothers don't have the opportunity to be away from the offspring for short periods, which in turn affects the quality of the natural caring behaviour of the mother towards offspring. Hence, AFR and NH should be considered as unique postnatal early life conditions.

Experimental condition	Abbreviation	Condition during the first postnatal weeks	Use
Brief maternal separation	BMS	Brief separations, 5-15 minutes	Simulates natural mother-infant relationship
Animal facility rearing	AFR	Conventional animal facility housing and handling. The mother and pup are constantly together.	Control for repeated handling and separations
Non-handling	NH	Left undisturbed in cage. No experimenter contact, and most often no cleaning of the cages	Used as control condition
Long maternal separation	LMS	Separations for longer periods of time, >180 min. Commonly 3-6 h daily	Simulates early-life stress/adverse events. Risk environment
Maternal deprivation	MD	Separations up to 24 h	Simulates early-life stress. Risk environment

Table 1. Experimental manipulations simulating postnatal stress/early life stress. Table modified from Nylander and Roman (2013).

Other animal models of early life stress can include long-lasting disruption of the mother-infant relationship (separation up to 24 h), also called maternal deprivation (see *Table 1*), which results in prolonged absence of maternal care (Levine & Wiener, 1988). A single 24-h maternal separation in the rat on PND 4, 9 or 18 has been shown to alter stress response and behaviour in adulthood (Lehmann et al., 1999; Lehmann et al., 2002). A different study found that maternal deprivation on PND 11 compared to AFR, led to lower corticosterone levels upon exposure to saline injections later in life (Suchecki & Tufik, 1997).

A more severe form of separation paradigm is isolation, where the offspring are exposed to daily separations from both mother and littermates. A recent rat study used isolation for 4 h each day, from PND 2-20 which according to authors mimics the situation of institutionalized children, and found that isolation increased vulnerability in female offspring

to develop anxiety-like behaviour later in life, compared to AFR offspring (Honeycutt et al., 2020).

A different approach to induce early life stress in the offspring is to manipulate the nesting and bedding material, which in turn results in altered behaviour of the mother towards the offspring. In this paradigm, the mother is provided with insufficient bedding and nesting material during early PNDs. This procedure stresses the mother, and maternal behaviour changes since the mother is forced to use her time to search for nesting material, instead of licking and grooming her offspring (Lewin et al., 2019). This fragmented maternal care can have a negative impact on the neural development in the offspring and increase depression- and anxiety-like behaviour in adulthood (Gallo et al., 2019).

Another model of early life stress relies on naturally occurring variations of active maternal care (licking and grooming and arched-back nursing). High levels of active maternal strengthen the regulation of the HPA axis in the offspring, effects which may be long-lasting (Liu et al., 1997). On the other hand, low levels of active maternal care have been found to alter the regulation of the HPA axis (Champagne et al., 2003) and lead to depression-like and anxiety-like behaviour later in life resembling the phenotypes found in humas who have been exposed to poor maternal care (Francis & Kuhar, 2008; Meaney, 2010).

1.1.4 Early life stress and later life stress

There exist two contradicting hypotheses regarding the development of psychopathology linked to early and later life stress. According to the “cumulative stress hypothesis» individuals are more likely to suffer from disease as stress and adversity accumulates through life (McEwen, 2003). Traumatic or adverse events early in life in

combination with later life stress can increase the vulnerability to develop psychiatric diseases (Nederhof & Schmidt, 2012). The other theory, the match/mismatch hypothesis, argues that disease and psychopathology is more likely to happen if a mismatch occurs between the early life environment and the later environment in adulthood (Nederhof & Schmidt, 2012). The early life environment has "programming" effects on later stress responses in adulthood. According to this hypothesis individuals will perform optimally when the later-life environment matches early life environment regarding stress levels (Santarelli et al., 2014). Evidence indicates that the increased stress responsivity later in life may be explained by alterations in the HPA axis after exposure to early life stress (Juruena, 2014). This is consistent with a study from 2018 including 70 women that had a history with early life stress, demonstrating that early life stress is related to disrupted physiological response to acute stress in adulthood, such as increased levels of cortisol (Kaiser et al., 2018).

Short-term stress can have positive effects on physiological and psychological processes, whereas stress experienced over a long period of time, chronic stress, have negative impacts on the same processes. There are very few animal studies addressing the impact of exposure to chronic stress in adulthood in animals previously exposed to early life stress such as maternal separation.

Chronic stress

The first animal model of chronic stress was developed by Katz in 1982 who subjected rats to a variety of relatively severe stressors after which the rats consumed less sucrose solution (sucrose was added to their drinking water) (Katz, 1982). This was interpreted as decreased response to rewards, which resembles a clinical core symptom of depression -

anhedonia. The model was later revised by Willner who developed the chronic mild stress (CMS) model (Willner et al., 1987). In contrast to severe stressors used by Katz, the CMS model is based on exposure to mild stressors and is a more realistic approach to mimic human everyday life hassles. In this protocol, rats or mice are exposed to repeated, unpredictable mild stressors, over a period of several weeks, resulting in the development of behavioural changes, including decreased response to rewards (Willner et al., 1992). CMS has also been shown to induce other changes associated with human depression such as changes in sleep and wakefulness (Cheeta et al., 1997; Willner, 2017). The CMS model is a highly validated animal model of depression, and it is an established translationally relevant model. A review and evaluation of the CMS model concludes that the model has good predictive validity as behavioural changes can be reversed by chronic treatment with a wide variety of antidepressants. The model also has face validity as almost all symptoms of depression have been demonstrated, as well as construct validity as CMS has been shown to cause a generalized decrease in responsiveness to rewards (Willner, 1997, 2017).

Few studies have examined the combination effect of early stress and chronic mild stress later in life. One study from Mrdalj and colleagues (2016) investigated how exposure to 4 weeks of CMS affected behaviour in adult male rats from different early life conditions; LMS, BMS and NH. Overall, exposure to CMS reduced sucrose preference in offspring from all three early life conditions but had different effect on other measures. After exposure to CMS, LMS offspring showed lower object exploration and enhanced pre-pulse inhibition and failed to show habituation to acoustic startle. On the other hand, exposure to CMS increased object exploration, increased pre-pulse inhibition and habituation to acoustic startle in BMS and NH offspring compared to offspring that had not been exposed to CMS (Mrdalj et al.,

2016). Another study from Mrdalj and colleagues (2014) in the same animals showed that exposure to CMS in adulthood led to lower body temperature in LMS and BMS offspring compared to baseline, but CMS exposure provoked a stronger and longer lasting hypothermia in LMS offspring compared to BMS offspring. The LMS offspring also showed greater sensitivity (lower body temperature) to specific stressors such as water- and food deprivation and wet bedding during the CMS protocol. Hence, these studies indicate that exposure to long-maternal separation can increase vulnerability to chronic mild stress in adulthood. Similar has been demonstrated in rat offspring after experience of low maternal care, where exposure to 7 weeks of CMS in adulthood increased levels of corticosterone and depression-like behaviour (reduced sucrose preference and locomotor activity) (Henningsen et al., 2012).

A more severe model of chronic stress, called chronic variable stress protocol, consists of random, intermittent, and unpredictable exposure to a variety of severe stressors. Such stressors can include restraint stress - which is a modified form of immobilization stress were the animals is placed in a plastic tube in order to block their movements (Au - Son et al., 2019) and social defeat – where male animals is placed in the cage of an aggressive male and is being physically defeated (Golden et al., 2011). In a study from Renard and colleagues it was found that chronic variable stress exposure led to lower secretion of corticosterone in LMS offspring, compared to AFR offspring (Renard et al., 2007). Chronic variable stress exposure has also been found to decrease responsivity to acute stress in LMS offspring (Ladd et al., 2005).

Clearly, early life stress can have profound effect on physiology and behaviour and influence stress susceptibility in adulthood. However, more studies are needed to address the impact of early life stress and later life stress on physiology and behaviour in adulthood.

1.2 Sleep and effects of stress on sleep

1.2.1 Sleep regulation

Sleep is a universal behaviour across animals (Greenspan et al., 2001), and can be defined as a "reversible behavioural state of perceptual disengagement from and unresponsiveness to the environment" (Carskadon & Dement, 2017). Many theories of the importance of sleep have been presented over the years. Sleep serves important physiological and psychological functions, such as maintaining a healthy immune system and metabolism, brain function and cognition (Greenspan et al., 2001; Krueger et al., 2016; Tononi & Cirelli, 2014) Sleep is thus important for good health and well-being and chronic sleep deprivation can be a potential risk for health problems such as obesity, immune dysfunction, and mood disorders such as depression (Medic et al., 2017).

To date, the most acknowledged model to describe sleep-wake regulation is the two-process model (Borbély, 1982; Borbély et al., 2016). The original version of the model was established to account for sleep regulation in humans (Borbély, 1982), and was later verified in rodents (Tobler et al., 1992). According to the model, sleep is regulated through a homeostatic factor (process S) and a circadian factor (process C) (see *Figure 1*). Process S represents the homeostatic need for sleep, or sleep pressure, which builds up during time spent awake, and declines during time spent in sleep (Borbély, 1982). The longer the prior awake period, the greater the build-up in sleep pressure. The sleep pressure is reflected in the amount of slow-wave activity (SWA; 0.5-4 Hz) during non-rapid eye movement (NREM) sleep, which is high during the early sleep period and decreases progressively throughout the night (Borbély & Achermann, 1999; Riedner et al., 2007) Increased SWA following sleep

deprivation has been demonstrated in humans and in rodents and is therefore one of the most important neurobiological markers of sleep pressure in mammals (Borbély et al., 1984; Leemburg et al., 2010).

Process C represents the endogenous circadian rhythm and promotes sleep and wakefulness at specific times of day (Borbély, 1982). Sleep usually occurs during the declining stage of the circadian rhythm, while it is difficult to fall asleep during the increasing stage of the circadian rhythm due to increasing activation (see *Figure 1*). The circadian rhythm is determined by a clock in the brain, located in the suprachiasmatic nucleus of the hypothalamus. This brain structure generates near 24-h rhythms independently of any environmental signals, working as a circadian pacemaker controlling the timing of many rhythmic behavioural, physiological, and metabolic functions, including temperature regulation, production of melatonin and cortisol (Monk et al., 1992). The term “circadian” rhythm is derived from Latin “circa” and “diem”, meaning “about a day", reflecting the length of about 24 h (Czeisler et al., 1999). Light is the most important time cue, also called zeitgeber, for entrainment of the circadian rhythm with environmental light/dark cycles (Gillette & Abbott, 2005). Other zeitgebers, such as timing of food intake and behaviour may also influence circadian rhythm.

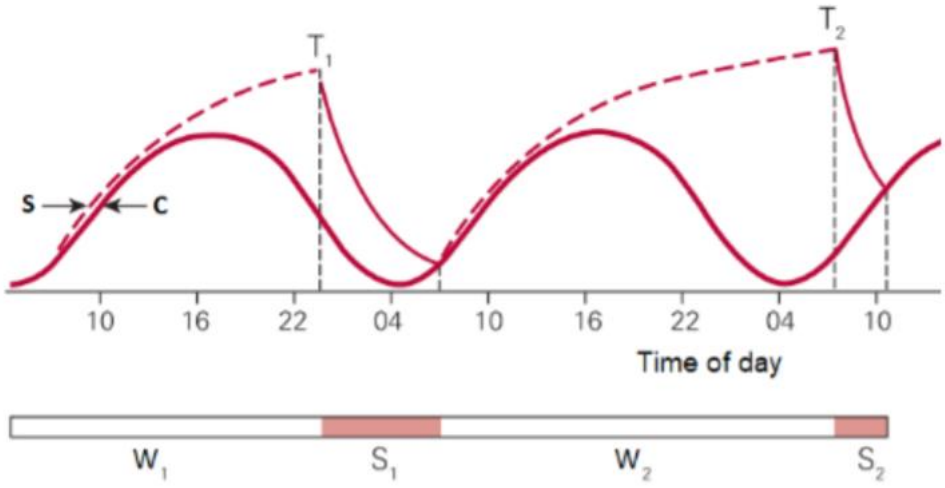

Figure 1. The two-process model of sleep regulation. Process S (dotted line) represents the homeostatic factor. Process C (red line) represents the circadian factor. The homeostatic need for sleep, sleep pressure, builds up during time spent awake (W1) and decreases during sleep (S1, highest at 23 PM, lowest at 7 AM in the figure). T1 represents when a human typically goes to sleep. Awakening would naturally happen when the sleep pressure is at its lowest, and when the circadian factor promotes wakefulness (where the two lines cross each other at 7 AM in the figure). T2 represents when a shift worker usually goes to sleep; in the increasing stage of the circadian oscillation. Figure modified from Grønli & Ursin (2009).

1.2.2 Sleep stages

Sleep is a complex and active process as brain activity changes during the course of sleep. In mammals, sleep is divided in two distinct types – non rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep (Moser et al., 2009). In humans, these stages alternate to form a NREM-REM cycle lasting 90-120 minutes (Carskadon & Dement, 2017). There is on average four to five sleep cycles during a typical 8-h sleep. Polysomnography (PSG) is considered a gold standard for sleep recording including recording of brain activity by electroencephalography (EEG), muscle activity by electromyogram (EMG) and eye movements by electrooculogram (EOG). Sleep staging is based on specific characteristics of EEG, EMG and EOG (American Academy of Sleep Medicine, AASM, 2007).

Wakefulness is a state of heightened levels of arousal and is characterized by desynchronized low amplitude and mixed frequency EEG activity, combined with high EMG.

In humans NREM sleep is divided into 3 different stages N1 - N3 (AASM, 2007). See *Figure 2*. Stage N1 indicates sleep onset and is referred to as drowsiness, or very light sleep. Usually, alpha activity (8-14 Hz) in wakefulness with eyes closed is replaced by theta waves (4-7 Hz) in N1. The EMG is usually decreased compared to wakefulness, and slow eye movements may occur in transition from wakefulness to N1 (Carskadon & Dement, 2017). Stage N1 usually comprises 5 percent of total sleep in adults. This stage is easily interrupted by noise. During stage N2 the arousal threshold for awakening is heightened. This stage is characterized by "sleep spindles" - short bursts of sigma waves (11-16 Hz, most commonly 12-14 Hz) lasting ≥0.5 seconds, and K-complexes – high amplitude and low-frequency delta waves (0,5-4 Hz). EMG may be equal or lower compared to N1. This stage lengthens with each cycle during the night, and accounts for about 50% of total sleep (AASM, 2007). During stage N3 the arousal threshold is at its highest and many environmental stimuli no longer produce any reactions in the sleeper during this stage. This stage is also referred to as deep sleep or slow wave sleep (SWS) in humans. Stage N3 is characterized by synchronized high amplitude slow wave activity known as delta waves (0,5-4 Hz) which account for 20% or more of the EEG signal (AASM, 2007). This stage comprises 15-25% of total sleep in adults. Deep sleep and delta waves are mostly present during the first period of the night and decreases as sleep pressure reduces during each sleep cycle. The first period of REM sleep occurs about 90 minutes after sleep-onset. REM sleep is characterized by desynchronized brain wave activity (low amplitude and mixed frequency), rhythmic theta activity, rapid eye movements and loss of muscle tonus (muscle atonia) with periodic twitches. REM sleep is also referred to as paradoxical sleep, as the brain waves characterising REM sleep are similar to wakefulness, but absence of muscle tonus clearly distinguishes REM sleep from

wakefulness. The periods of REM sleep become progressively prolonged as the sleep episode progresses (Carskadon & Dement, 2017). Dreaming is most often associated with REM sleep even though dreaming also occurs in NREM sleep. REM sleep comprises about 20-25% of total sleep in adults.

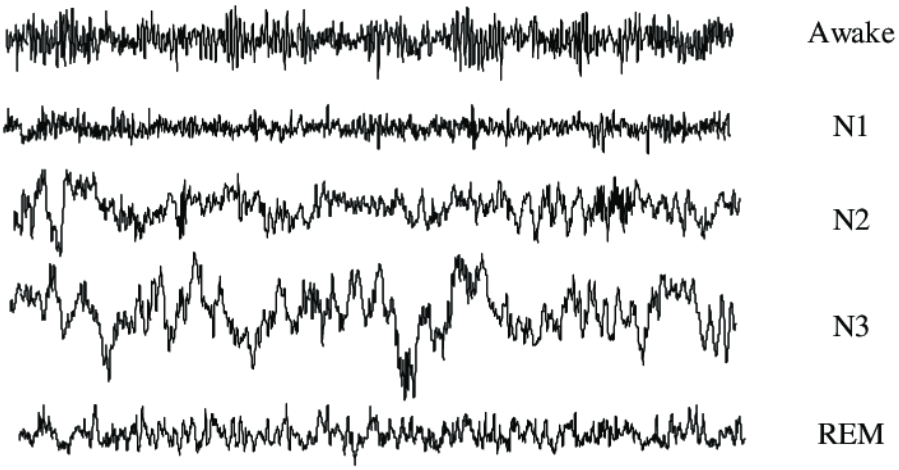

Figure 2. EEG waves representing the different sleep-wake stages in humans; wakefulness, NREM sleep (N1-N3) and REM sleep. Figure by Khalighi et al., 2013.

In rats, one differentiates between wakefulness, two stages of SWS sleep (NREM sleep) and REM sleep. See *Figure 3.* Wakefulness is characterized by synchronized low amplitude and high-frequency beta (15-30 Hz) and gamma (30-120 Hz) EEG activity, combined with high muscle tonus (EMG) (Neckelmann & Ursin, 1993). SWS includes both light and deep sleep, SWS1 and SWS2 respectively. Light sleep, or SWS1 is characterized by sleep spindles (11-16 Hz) and less than 50 % of high amplitude delta waves (0,5-4 Hz). Deep sleep, or SWS2 is characterized by sleep spindles and 50 % or more of delta waves. REM sleep is characterized by theta EEG activity (6–9 Hz), rapid-eye movements and muscle atonia with periodic muscle twitches (Neckelmann & Ursin, 1993). Brief periods with

wakefulness occur during both SWS and REM sleep. A sleep cycle in rats lasts approximately 10-12 minutes. Whereas humans usually have monophasic sleep (one sleep period of about 8 h a day) (Simasko & Mukherjee, 2009), rats are polyphasic sleepers, i.e., they have multiple periods of sleep during 24 h. See *Figure 4.* In contrast to humans, rats are nocturnal animals and mainly awake and active during the night (active phase) although they have short periods of sleep during the night. They sleep mostly during daytime (inactive phase) with short periods of wakefulness (Borbély, 1975).

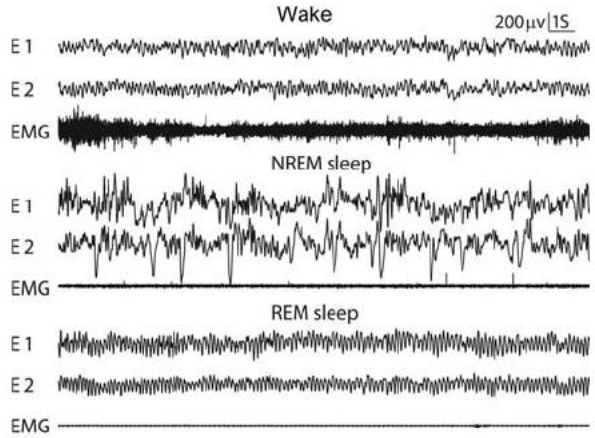

Figure 3. Electroencephalogram illustrating a rat' s brain activity during wake, NREM sleep (SWS1 and SWS2) and REM sleep. E1 and E2 illustrates the placement of the two electrodes with E1 showing brain-activity from frontal-parietal lobe and E2 showing brain activity from parietal-occipital lobe. Figure modified from Fang et al. (2009).

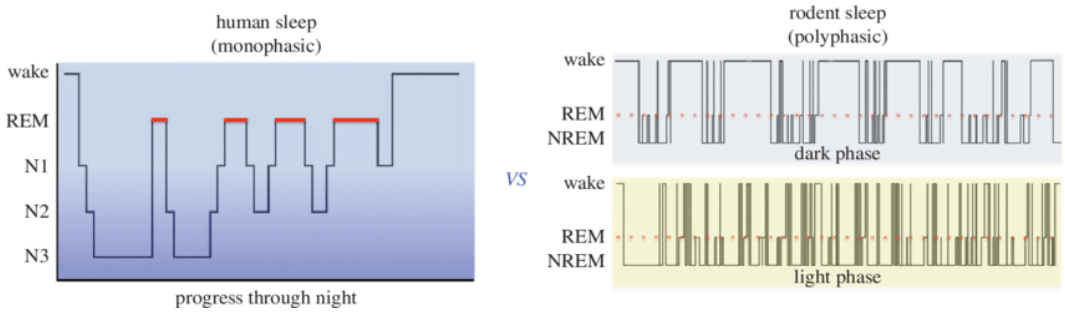

Figure 4. Comparisons of hypnograms representing monophasic sleep pattern in humans (left) and polyphasic sleep pattern in rodents (right) during light- and dark phase. REM sleep defined with red marks. N1-N3: non-rapid eye movement sleep/NREM sleep. Figure by Mong & Cusmano (2016).

1.2.3 Effects of early life stress on sleep

The postnatal period is important for normal brain development across species, and environmental manipulations during this period have been shown to have significant impact on sleep later in life. In humans, it has been shown that high stress load during childhood might be a vulnerability factor for sleep continuity problems and increase the risk of self-reported sleep disturbances in adulthood (Chapman et al., 2011; Duval et al., 2013; Steine et al., 2012). Few studies have used objective sleep recording to examine changes in sleep architecture in humans that have experienced early life stress. In one study early life stress was associated with shortened actigraphically assessed total sleep time, prolonged sleep onset latency, decreased sleep efficiency and an increased number of body movements in sleep in adulthood (Schäfer & Bader, 2013). In a different study PSG and actigraphy was used to assess sleep architecture in patients aged 21-55 years with primary insomnia and self-reported early life stress. Individuals reporting high levels of early life stress had disturbed sleep with increased number of awakenings and more movement arousals, as well as more light sleep (N2) and less deep sleep (N3) compared to individuals reporting low levels or no experience of early life stress (Bader et al., 2007).

Most studies on early life stress focus on postnatal stress, but there is evidence for sleep-changes in adulthood after prenatal stress, as well. Maternal stress such as experiencing high anger, anxiety or depression during pregnancy may result in long-lasting modifications in stress reactivity in the child which may persist into adulthood and contribute to the development of chronic insomnia and maladaptive stress responses later in life (Palagini et al., 2015).

Animal studies have also investigated how early life stress impacts sleep, first by Reite and Snyder in bonnet macaque monkeys. Ten days of maternal separation (away from mother and social group, but left with other adult individuals) increased nightly awakenings, and time spent in REM sleep in the macaque offspring (Reite & Snyder, 1982).

Several studies have investigated the effect of maternal separation on sleep in male rats. One study from Tiba and colleagues found that LMS (3 h a day) and BMS (15 min a day) during the first two postnatal weeks, induced long-lasting changes in sleep architecture, with LMS offspring showing an increased time spent in REM sleep and had more REM sleep episodes compared to BMS and NH offspring in adulthood. These differences were observed during the rats' inactive phase, whereas for the active phase there were no differences in sleep and wakefulness between the three early life conditions (LMS, BMS, NH) (Tiba et al., 2004). In the study from Mrdalj and colleagues (2013) LMS (3 h daily) during PND 2-14 led to changes in quality of SWS during inactive phase, as LMS offspring displayed less light sleep (SWS1) and more deep sleep (SWS2) compared to BMS offspring. LMS offspring also showed lower power of delta waves in SWS (0.5-4 Hz), and a slower reduction of sleep pressure compared to BMS offspring. The study did not investigate sleep and wakefulness during the offspring's active phase (Mrdalj et al., 2013).

A study from Feng and colleagues demonstrated that 10 days exposure to a more severe form of LMS (3 h, 2 times a day) during early PNDs resulted in reduced total sleep time, and increased time spent in wakefulness during the inactive phase compared to handled offspring (separated from the mother, but immediately returned back to cage) when the rats were at 3 months of age (Feng et al., 2007). In a 2012 study from Feng et al., also using the same experimental conditions, exposure to LMS (3 h, 2 times a day) from PND 4-14 led to

reduced REM sleep and increased time spent in wakefulness were significantly increased during inactive phase compared to handled offspring, consistent with findings from the 2007 study (Feng et al., 2012). In both studies the differences observed were during the rats' inactive phase, whereas for the active phase there were no differences in sleep and wakefulness between the groups (Feng et al., 2012; Feng et al., 2007).

In a study from Sampath et al., male rats were subjected to a more severe form of maternal separation during PND 5-7 (LMS 6 h daily in isolation - separated from both mother and littermates). At 2 months of age, LMS offspring spent increased time in REM sleep, and had more total sleep time compared to NH offspring (Sampath et al., 2014). However, it is not clear from the study whether these changes are observed in the rats' inactive phase or active phase.

Other forms of environmental manipulations to induce early life stress have also been found to affect sleep and behaviour later in life. Lewin and colleagues used the limited nesting and bedding paradigm to induce early life stress from PND 8-12. They found disruptions in sleep during inactive phase but not active phase, including SWS fragmentation (more episodes but shorter episode duration) and decreased number of sleep spindles in the offspring later in life, compared to offspring from control condition (Lewin et al., 2019). In a study with a different approach to early life stress, rats that were cross-fostered (i.e. offspring were changed between mothers), showed changes in sleep architecture in adulthood with increased episode duration of SWS and REM sleep during inactive phase, and increased number of REM sleep episodes during both active and inactive phase, compared to offspring that were not cross-fostered (Santangeli et al., 2016).

Taken together, results from these studies indicate that early life stress affects sleep in multiple ways across species, and that these changes can be lifelong.

1.2.4 Effects of chronic stress in adulthood on sleep

Polysomnographic evidence concerning stressful life events and sleep shows that psychosocial stress or daily hassels experienced by humans affect sleep, including changes such as increased amount of REM sleep, decreased REM sleep latency and reduced amount of deep sleep (N3) (Kecklund & Akerstedt, 2004; Kim & Dimsdale, 2007). It is possible that different types of stressors as well as the chronicity and intensity of the stressor may impact sleep differently (Kim & Dimsdale, 2007). It is well known that chronic stress is associated with increased risk for depression and anxiety, and such risk may be mediated through altered sleep (Mariotti, 2015).

Animal studies addressing the impact of chronic stress on sleep are scarce. Exposure to chronic mild stress (CMS) later in life in rats have been shown to increase REM sleep and sleep fragmentation. In a study from Grønli and colleagues the rats that underwent the CMS protocol had an increased number of arousals during sleep, as well as the duration of sleep stage episodes was decreased compared to baseline recording. The largest effect was obtained after 2 weeks of CMS protocol (Grønli et al., 2004). In another study, the effects of stress were maximal following 21 days of CMS, where the animals demonstrated decreases in deep sleep and wakefulness, as well as increased time spent in REM sleep, and reduced latency to the onset of the first REM sleep period (Cheeta et al., 1997).

1.2.5 Effects of early life stress and later life stress on sleep

As noted earlier, few studies have investigated effects of early life stress on sleep, and even fewer studies have investigated effects of early life stress in combination with stress exposure in adulthood on sleep. In the study from Tiba and colleagues (2004), male rat offspring from LMS (3 h daily), BMS (15 min daily) and NH early life condition were exposed to 1 h of cold stress (4 degrees) in adulthood. In the first 2 h following cold stress (inactive phase) all offspring showed reduced time spent in REM sleep, whereas only NH offspring showed reduced time spent in SWS (Tiba 2004). In a different study from Tiba and colleagues (2003), BMS and NH offspring were exposed to 1 h of restraint stress. In the first 3 h following restraint stress (inactive phase), both groups showed reduced time spent in SWS and REM sleep, and increased number and duration of wakefulness episodes. The study, however, did not include an LMS condition (Tiba et al., 2003).

Only one study have investigated consequences on sleep after CMS exposure (4 weeks) in adulthood in male rats that have been exposed to long- and brief-maternal separations at PND 2-14 (Mrdalj et al., 2013). Two days after ended CMS protocol, both LMS and BMS offspring showed a decrease in total sleep time and decrease in SWS2 (deep sleep), and an increase in time spent in wakefulness. However, CMS did affect sleep differently in the two early life conditions. LMS offspring had more total sleep time, spent less time in wakefulness, and had more episodes of REM sleep and higher percentage of SWS episodes ending in REM sleep compared to BMS offspring. In a different study from Mrdalj and colleagues (2014) it was investigated how CMS exposure affected body temperature, heart rate and locomotor activity in LMS and BMS offspring. Two days after ended CMS protocol offspring from both conditions displayed lower body temperature (hypothermia). However,

this effect was stronger, and lasted longer in the LMS group, compared to BMS group. The study also investigated how LMS and BMS offspring reacted to specific mild stressors during the second week of CMS protocol and found that some of the stressors provoked a stronger response in the LMS compared to BMS offspring. During the stressors “water deprivation”, “food deprivation” and “wet bedding”, all of which were presented during 12 h active phase, LMS offspring displayed lower body temperature compared to BMS offspring. There was also a tendency toward lower body temperature during stressors “social stress” (2 h paired caging in inactive phase) and during “continuous light” (in active phase) in LMS compared to BMS offspring. For heart rate and locomotor activity during the different stressors there were no effects of group, even though the stressor “water deprivation” showed a tendency toward a lower heart rate in the LMS offspring compared to BMS offspring (Mrdalj et al., 2014). The study did not investigate how different mild stressors affected sleep and wakefulness.

It follows that more studies are needed to address how early life stress in combination with stress exposure in adulthood affect sleep, and specifically how different stressors affect sleep during exposure to CMS. The present study is the first to examine how different mild stressors affect sleep and wakefulness during exposure to CMS.

1.3 Aims of the study and hypotheses

The first aim of the study is to investigate the effects of early life stress on sleep and wakefulness during both active and inactive phase. To model early life stress LMS condition was used and compared to BMS condition. Further aim is to investigate how one week of chronic mild stress in adulthood will affect sleep and wakefulness during stress exposure, in both active and inactive phase, in rats from different early life conditions (LMS, BMS). One

specific aim is to investigate if different individual mild stressors during CMS will affect sleep and wakefulness differently in LMS and BMS offspring.

The following hypotheses were defined for sleep and wakefulness in LMS and BMS offspring:

- During 12 h active phase LMS compared to BMS offspring, will display similar time spent in wakefulness, SWS and REM sleep and similar total sleep time, as well as similar number and duration of wakefulness, SWS and REM sleep episodes.
- During 12 h inactive phase LMS compared to BMS offspring, will display similar time spent in wakefulness, SWS and REM sleep and similar total sleep time, as well as similar number and duration of wakefulness, SWS and REM sleep episodes.

The following hypotheses were defined for sleep and wakefulness in adult LMS and BMS offspring during 1 week of CMS:

- Across 7 days of CMS during active phase, both LMS and BMS offspring will show decreased time spent in wakefulness and increased total sleep time; and LMS compared to BMS offspring will show less time spent in wakefulness and more total sleep time.
- Across 7 days of CMS during inactive phase, both LMS and BMS offspring will show increased time spent in wakefulness and decreased total sleep time; and LMS

compared to BMS offspring will show less total sleep time as well as increased number of REM sleep episodes.

- Individual mild stressors will affect sleep and wakefulness differently in LMS and BMS offspring, this is expected in particular during stressors: water deprivation, continuous light, food deprivation, social stress, and wet bedding.

2. Methods

2.1 Ethical considerations and approval

The experiments presented in this thesis have been approved and registered by the Norwegian Animal Research Authority (Permit Number: 07/9421-2007025) and conducted in accordance with Norwegian laws and regulations controlling experiments in live animals (Forskrift om bruk av dyr i forsøk, 2015).

The principles of the "3R's": reduction, refinement, and replacement (Russel & Burch, 1959) have been applied during the experiment. The number of animals was reduced to a minimum, whilst still providing sufficient data for statistical analysis. Only adult, male rats already available at the animal facility were used for mating, while female offspring were assigned to a different study. Refinement was addressed by using a wireless EEG signal recording that allows for the animals to be left undisturbed in their home cages. Additional parameters such as heartrate, activity and body temperature were also measured in the same animals and published in a different study (Mrdalj et al., 2014). This limited the use of additional animals. Every effort was made to minimize suffering of the rats. The rats were given hypnorm-dormicum anaesthesia during surgery, and antibiotics post-surgery to reduce the risk of inflammation and discomfort. At the present time, it is not possible to use *in vitro*

methods to study the impact of early life stress on psychological function and behaviour, and therefore *replacement,* or the use of an alternative method cannot replace *in vivo* experiments.

2.2 Research design

The experiment was conducted at the Department of Biological and Medical Psychology, University of Bergen, in the spring of 2009 as a part of a PhD project (Mrdalj et al., 2013). The study consisted of exposure to early life stress, weaning, implantation of transmitters/surgery and exposure to chronic mild stress (see *Figure 5*).

During the maternal separation experiment, new-born Wistar rats (NTac:WH) were daily exposed to either brief maternal separation (BMS) of 10 min or long maternal separation (LMS) of 3 h, during PND 2-14. The offspring were left undisturbed after PND 14 and until weaning took place at PND 22. At this point offspring were separated by gender and group housed in IVC type IV cages. At 9 weeks of age the offspring were implanted with telemetric devices, and then housed individually. A baseline recording was done 24 h prior to the CMS protocol. CMS protocol started at PND 90 and lasted for 4 weeks. Telemetric recordings using electroencephalography (EEG) and electromyography (EMG) were conducted in both BMS and LMS offspring.

2.3 Animals and housing procedures

A total of 16 male outbred rats (Wistar, NTac:WH, Taconic, Denmark) were used in the experiment. One animal from the BMS group died before data collection, due to postoperative complications. Two animals were further excluded due to artefacts in the EEG signals. This study therefore includes 6 BMS and 7 LMS male offspring. The rats were kept at

a 12 h light/12 h dark schedule, with 1 h gradual increase with lights fully on at 07.00 A.M. From first postnatal day (defined PND 0) – PND 22 the offspring were group housed in individually ventilated system cages (IVC system, Tecniplast ®, Italy) of type IV (480 x 375 x 210 mm, floor area 1400 cm^2) at an ambient temperature of 22 ±1 °C and air humidity of 52 ± 2%). After weaning at PND 22 the offspring were housed in IVC type IV cages in groups of 4-5 of same gender. After surgical procedure (telemetric implanting) at PND 56 the offspring were single housed in individual cages; type III cage (425 x 266 x 185 mm, floor area 800 cm^2).

All animals had *ad libitum* access to food (Standard rodent diet, Rat and Mouse 1 (RM1), Special Diets Services, Witham, Essex, England) and water, except when tested for sucrose intake and during food or water deprivation as part of the CMS protocol.

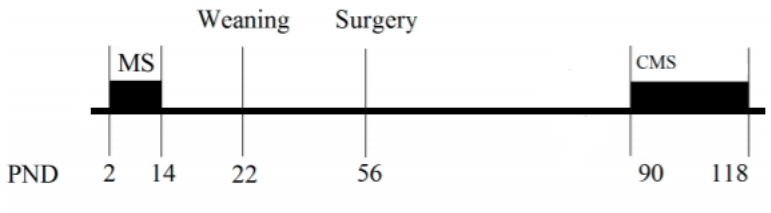

Figure 5. Timeline of experimental design. Maternal separation (MS); brief maternal separation (BMS) or long maternal separation (LMS) were performed during postnatal days (PND) 2-14. Weaning took place at PND 22. Surgery for telemetric recording was conducted at PND 56. CMS exposure started at day 90 and lasted until day 118, in total; 4 weeks (illustrated in the black bar). Figure modified from Mrdalj et.al (2013).

2.4 Early life conditions

2.4.1 Long maternal separation

The day of birth was defined as postnatal day (PND) 0. From PND 2-14, the LMS offspring were separated from their mothers daily for one period of 180 min starting at 09:00 A.M. The mother was removed from its home cage and placed in a separate cage, a type III cage, with *ad libitum* access to food and water. The mother was placed in a different room adjacent to the offspring. Each litter were moved to a cage and divided with cardboard compartments, with wood shavings/sawdust as floor bedding (a cubicle for each litter). Artificial heating was provided to the pups. The temperature was regulated and monitored (PND 2-7:32-34°C, PND 8-14:28-30 °C). The offspring and the mother were reunited in reverse order at the end of each daily separation. The same compartments and cages were used daily, and none of the cages were cleaned during the procedure (Mrdalj et al., 2013).

2.4.2 Brief maternal separation

From PND 2-14, the BMS offspring were separated from their mothers daily for one period of 10 min starting at 09:00 A.M. Artificial heating was not provided for the BMS offspring, due to an earlier report stating that offspring can maintain their body temperature for up to 30 min in the absence of the mother (Suchecki et al., 1993). The offspring were left undisturbed after maternal separation at PND 14 and until weaning took place at PND 22.

2.5 Chronic mild stress protocol

The CMS protocol used in this study was modified from (Willner et al., 1987) and (Grønli et al., 2004), and some additional stressors were included (exposure to a cage without

bedding for 21 h, followed by 3 h exposure to 3 cm of water in the same cage). See *Table 2* for an overview of the CMS protocol. At PND 90, the LMS and BMS offspring were exposed to chronic mild stressors for 4 weeks. The CMS protocol consisted of exposure to one or more mild stressors each day of the 4-week period. The stressors were presented both during the rats' active phase and inactive phase. The CMS protocol consisted of 11 mild stressors, varying in intensity and duration. Each CMS week included: exposure to food deprivation for 18 h followed by 1 h of restricted access to food (4-5 food pellets à 45 mg, formula P (P.J. Noyes Company Inc, Lancaster)), two periods of water deprivation (16 h and 20 h) followed by exposure to an empty water bottle (1 h), exposure to 20 h of wet bedding (20 h; bedding soaked with 300 ml water at room temperature), two 3 h periods with tilted cage at 45 degrees angle, one exposure to social stress (two male rats paired per cage, 2 h. To prevent the development of persistent dominant behaviour, rats alternated between being the resident and the intruder), one continuous light period of 36 h. New stressors included were new cage without bedding (21 h) followed by 3 cm water in cage (3 h). At the beginning of each week, the rats were exposed to 1% sucrose solution preceded by 4 h of food and water deprivation, however sucrose data are not included in the present study.

CMS protocol

Chronic mild stress protocol.

Stressor	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday	Sunday
Sucrose consumption test			16:00→17:00				
Food deprive	09:00		12:00→16:00				15:00→
Restricted food	09:00→10:00						
Water deprive			12:00→16:00, 17:00→	10:00	13:00→	10:00	
Empty bottle				09:00→10:00		09:00→10:00	
Tilted cage		10:00→13:00		10:00→13:00			
Paired caging	13:00→15:00						
Wet cage			17:00→	13:00			
New cage no bedding				13:00→	10:00		
Water in cage					10:00→13:00		
Bedding in cage					13:00		
Continuous light						07:00→	19:00

Table 2. Chronic mild stress protocol. Table from Mrdalj et al. (2013).

2.6 Surgical procedure

In order to continuously and wirelessly monitor electroencephalogram (EEG) and electromyogram (EMG) throughout the experiment, a wireless transmitter (4ET, Physiotel®, Data Sciences International) was implanted subcutaneously (s.c) in LMS and BMS offspring at PND 56. Prior to surgery, the rats were anaesthetized with an injection of a mixture of fentanyl 0.277mg/kg animal, fluanizone 8.8 mg/kg and midazolam 2.5 mg/kg (Hypnorm, Janssen; Dormicum, Roche). The rats were positioned in a stereotaxic apparatus (Kopf®, USA) with the head fixed with incisor bars. The effect of anaesthesia was monitored during the whole procedure and additional anaesthesia was given at approximately 45 min intervals. Surgical incisions were made over the lower back, head, and neck, and along the shoulder muscle. The transmitter was placed in a “saddleback” position and placed in an s.c pocket.

Two electrodes were attached to the skull for EEG recordings and two electrode pairs were attached to the neck muscle for EMG recording. The EEG leads were placed in frontal-frontal (FF) and frontal-parietal (FP) position with the following coordinates; 2 mm anterior to bregma and 2 mm lateral to the midline for the frontal electrodes, and 2 mm anterior to lambda and 2 mm lateral to the midline for the posterior electrode (Neckelmann & Ursin, 1993). See *Figure 6* for placement of electrodes. The skin was closed with interrupted mattress sutures. To reduce postoperative discomfort and pain and to minimize the risk of infection/inflammation, the rats were given antibiotics in their drinking water (Bactrim, 5 ml per 250 ml of water) one day before the surgery and two days post-surgery. Analgesia was given twice a day for 3 days post-operation. The rats were allowed 14 days postoperative recovery and time to regain their postoperative weight. The rats received care every day throughout this period.

One animal from the BMS group died before data collection due to postoperative complications.

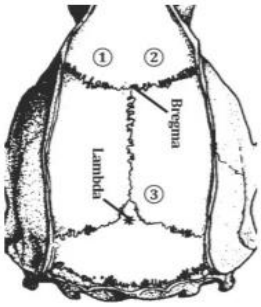

Figure 6. Placement of electrodes for electroencephalographic recording. 1: reference electrode, 1 + 2: frontal-frontal (FF) derivation, 1 + 3: frontal-parietal (FP) derivation. Bregma is the intersection of the coronal suture and the sagittal suture, while lambda is the anatomical point in the intersection between the sagittal suture and the lambdoid suture. The illustration is modified from Paxinos & Watson (1998).

2.7 Telemetric recording

To initiate wireless telemetric recording, a magnet was passed along the animal's side at the site of the implanted battery. Signals were collected with receiver type RPC-2 (Data Sciences International) placed directly under the home cage. The receiver was connected to a data exchange matrix where signals were converted and transferred to the acquisition software Dataquest ART (version 4.1, Data Sciences International). Sampling frequency was set to 250 Hz for both EEG- and EMG signals. All animals remained undisturbed in their home cages during recordings.

2.8 Criteria for sleep scoring

Sleep and wake stages were scored manually offline using NeuroScore™ software (version 3.0., Data Sciences International). Three distinct stages were scored in 10 s epochs: wakefulness, slow-wave sleep (SWS) and rapid-eye movement sleep (REM sleep). Epochs were scored across the entire sleep-wake cycle, during the 12 h active/dark phase (7:00 PM – 7:00 AM) and during the 12 h inactive/light phase (7:00 AM – 7:00 PM). EEG and EMG data during one continuous week of CMS (week 2) were analysed using Neuroscore. For visual scoring procedure FF-EEG signals were filtered with high-pass frequency at 3Hz and low-pass frequency at 35 Hz. FP-EEG signals were filtered with high-pass frequency at 0.5 Hz and low-pass frequency at 35 Hz. To remove low-frequency artefacts, the EMG signal was high-pass filtered at 5 Hz. Epochs with artefacts in more than >50% of the 10 sec epoch, were excluded.

Sleep stages were scored based on specific EEG and EMG criteria developed by Neckelmann and Ursin (1993). Before assigning the stage to a given 10-s epoch the criteria

for a given stage needed to be fulfilled in ≥50% of the epoch. Wakefulness was scored when there was high-frequency low voltage activity in EEG channels and high to moderate activity in the EMG channel. SWS was scored when spindle activity (11-16 Hz) was present in the FF channel, and with delta (0.5-4 Hz) activity in the FP channel, and EMG was reduced compared to wakefulness. It was not differentiated between light sleep (SWS1) and deep sleep (SWS2) was scored as one; SWS. REM sleep was scored when theta (6-9 Hz) waves were dominant in the FP channel and EMG activity was reduced to its lowest or abolished (muscle atonia).

To assess the duration of wakefulness and different sleep stages a customized MATLAB based application, SLEEP Report, developed by Professor J. Wisor at Sleep and Performance Research Center, Washington State University, was used to process the manually scored sleep data from Neuroscore. The algorithm in MATLAB allows us to extract detailed information about the different sleep stages and the following parameters were extracted; total length (in minutes) and number and duration of different wakefulness and sleep episodes. This script creates an output file for each rat based on the script analyses, that can easily be imported in statistical analysis software for further analysis.

2.8.1 Quality assessment of sleep scoring

Inter-rater reliability was calculated to assess quality of sleep scoring. Sleep scoring of 12 h inactive phase in the present study was compared with sleep scoring of 12 h inactive phase performed by Mrdalj and colleagues in 2013. Cohen's kappa measures the agreement between two raters. If the raters are in complete agreement, then $\boldsymbol{k} = 1$. If there is no agreement among the raters other than what would be expected by chance, then $\boldsymbol{k} = 0$

(McHugh, 2012). The kappa value was $\boldsymbol{k}$ = 0,87±0,03 for wakefulness, 0,89±0,02 for SWS and 0,92±0,02 for REM sleep. The overall percentage agreement was 93,95%±1,04%.

2.9 Statistical analyses

All data were acquired from 7 LMS and 6 BMS offspring. Statistica (version 13.3 StatSoft Inc) was used to perform statistical analyses. Significance was accepted at $p \leq 0.05$.

Effect of early life stress: One-way analysis of variance (ANOVA) was used for 12 h interval during active phase and 12 h interval during inactive phase and with "early life stress" (LMS, BMS) as independent variable, for time spent in wakefulness, SWS and REM sleep and total sleep time, and for number of episodes and mean episode duration of wakefulness, SWS and REM sleep. Repeated measures ANOVA was used to analyze 3 h intervals during inactive phase and 3 h intervals during active phase, with "early life stress" (LMS, BMS) as the independent variable, and "interval" as repeated measure, for time spent in wakefulness, SWS and REM sleep and total sleep time.

Effect of CMS: For 12 h active and 12 h inactive phase during 7 days of CMS, a repeated measures ANOVA was used to make comparisons between the different days during CMS and baseline. "Early life stress" (LMS, BMS) was used as the independent variable, and "day" was used as the repeated measure for time spent in wakefulness, SWS and REM sleep and total sleep time, and for number of episodes and mean episode duration of wakefulness, SWS and REM sleep. Repeated measures ANOVA was then used to analyze 3 h intervals during active phase and 3 h intervals during inactive phase, with "early life stress" (LMS, BMS) as the independent variable, and "day" and "interval" and as repeated measures, for time spent in wakefulness, SWS and REM sleep and total sleep time.

Effect of individual stressors during CMS: To investigate the impact of specific stressors (wet bedding, food-deprivation, water deprivation and continuous light) on time spent in wakefulness, SWS and REM sleep, and total sleep time repeated measures ANOVA was performed for 3 h intervals with ‘‘early life stress’’ (LMS, BMS) as independent variable and ‘‘interval” and “day” as repeated measures. To investigate the impact of specific stressors (social stress and water in cage) on time spent in wakefulness, SWS and REM sleep, and total sleep time, repeated measures ANOVA was performed for 3 h intervals with ‘‘early life stress’’ (LMS, BMS) as independent variable and “day” as repeated measures. Analysis during social stress were done for 3 h as the start and ending of stress exposure differed with several minutes between the rats, even though the actual stress exposure were no longer than 2 h for each rat.

All significant ANOVA effects were further tested with Fisher’s least significant difference post-hoc test.

3.0 Results

All results are given as mean and standard deviation (±SD), except for *Figure 7* where data are shown as mean and standard error of the mean.

3.1 Effect of early life stress on sleep and wakefulness

Distribution of time spent in wakefulness (panel A), SWS (panel B) and REM sleep (panel C) in LMS and BMS offspring across one 12 h active phase and one 12 h inactive phase is shown in the first part of *Figure 7* (baseline; data are displayed in 1 h intervals).

Active phase

Analysis of 12 h interval

Analysis of 12 h interval during active phase showed no effect of early life stress on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(1,11)}$≤2,3; p's≤0,91) and there was no effect of early life stress on number of episodes or mean episode duration for either wakefulness, SWS or REM sleep (all F's$_{(1,11)}$ ≤3,2; p's≤0,87). See *Table 3*.

Analysis of 3 h intervals

Analysis of 3 h intervals during active phase showed no effect of early life stress on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(1,11)}$≤2,3; p's≤0,91). There was an effect of interval on all parameters (all F's$_{(3,33)}$≤8,5; p's≤0,05). Rats are nocturnal animals, and therefore spend more time in wakefulness during the night which is their active phase. As expected, during most of the active phase, in interval 1 (19.00-22.00), interval 2 (22.00-01.00) and interval 3 (01.00-04.00), all offspring (LMS, BMS) showed more

time in wakefulness and less time in SWS and REM sleep and had less total sleep time compared to the last interval (04.00-07.00). See *Table A* in Appendix.

There was a significant interaction between early life stress and interval on time spent in wakefulness and SWS, and total sleep time (all F's$_{(3,33)}$≤3,1; p's<0,05), but no interaction effect for REM sleep ($F_{(3,33)=}$1,5; p=0,24). During interval 1 (19.00-22.00) LMS group compared to BMS group, spent less time in wakefulness (122,81±16,71 min vs 144,22±9,38 min; p<0,05) (see *Figure 7,* panel A), and had more total sleep time (56,33±17,09 min vs 34,78±8,96 min; p<0,05). During interval 2 (22.00-01.00) LMS group compared to BMS group, spent more time in wakefulness (148,62±15,36 min vs 126,67±8,90 min; p<0,05) (see *Figure 3*, panel A), less time in SWS (29,02±13,72 min vs 49,31±7,24 min; p<0,05) (see *Figure 3*, panel B) and had less total sleep time (30,33±15,52 min vs 52,89±9,30 min; p<0,05).

Inactive phase

Analysis of 12 h interval

Analysis of 12 h interval during inactive phase showed no effect of early life stress on time spent in wakefulness, SWS or REM sleep, or total sleep time, (all F's$_{(1,11)}$≤0,4; p's≤0,96) and there was no effect of early life stress on number of episodes or mean episode duration for either wakefulness, SWS or REM sleep (all F's$_{(1,11)}$ ≤1,0; p's≤0,4). See *Table 4*.

Analysis of 3 h intervals

Analysis of 3 h intervals during inactive phase showed no effect of early life stress on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(1,11)}$≤0,4; p's≤0,96). There was an effect of interval for all parameters (all F's$_{(3,33)}$≤29,8; p's<0,001). Rats are

nocturnal animals, and hence it is typical that they spend more time in sleep during daytime which is their inactive phase. As expected, during most of the inactive phase, in interval 1 (07.00-10.00), interval 2 (10.00-13.00) and interval 3 (13.00-16.00), all animals spent more time in both SWS and REM sleep, and had more total sleep time, and less wakefulness, compared to the last interval (16.00-19.00). See *Table A* in Appendix.

There was no significant interaction between early life stress and interval on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(3,33)}$≤2,4; p's≤0,93).

3.2 Effect of CMS on sleep and wakefulness

Data for 12 h intervals during baseline and each of the CMS days are presented in *Table 3* (active phase) and *Table 4* (inactive phase).

Active phase

Time spent in wakefulness and sleep

There was an effect of CMS on time spent in wakefulness, SWS, and REM sleep, and total sleep time ($F_{(7,77)}$≤31,3; p<0,001). During 12 h active phase on each day of CMS, all animals spent less time in wakefulness, and spent more time in SWS and REM sleep and had more total sleep time compared to 12 h active phase during baseline (all p's<0,001). See *Table B* in appendix. There was no interaction effect of early life stress and CMS on time spent in wakefulness, SWS, or REM sleep, or total sleep time (all F's$_{(7,77)}$≤1,4; p≤0,45), see *Table 3*.

Wakefulness episodes:

There was an effect of CMS on number of wakefulness episodes and mean episode duration (all F's$_{(7,77)}$≤13,6; p's<0,001). Compared to baseline, all animals had more, but shorter episodes of wakefulness during active phase on CMS day 2, 3, 5 and 6, in addition to shorter episode duration on day 1 (all p's≤0,01). See *Table C* in Appendix. There was also an interaction effect of early life stress and CMS on mean episode duration ($F_{(7,77)=}$2,3; p<0,05), but not for number of episodes ($F_{(7,77)=}$1,6; p=0,13). See *Table 3*. BMS group had shorter episodes of wakefulness on CMS day 1, 2, 3, 5 and 6 compared to their own baseline (all p's≤0,05). LMS group had shorter episodes of wakefulness on CMS day 2, 3, 5, 6 and 7 compared to their own baseline (all p's≤0,05). Compared to BMS, LMS group also showed shorter episodes of wakefulness on CMS day 4 and 7 (all p's≤0,05).

Active Phase 19:00-07:00	Baseline			CMS Day 1			CMS Day 2			CMS Day 3		
Sleep stages:	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*
Wakefulness	513,91 (36,62)	496,94 (14,89)	ns	396,19 (28,06)	376,47 (33,40)	ns	377,36 (23,30)	388,31 (49,44)	ns	336,52 (24,38)	322,31 (32,51)	ns
SWS	179,12 (29,46)	198,83 (12,37)	ns	244,76 (18,26)	264,94 (26,08)	ns	281,10 (17,69)	279,42 (39,30)	ns	323,57 (25,15)	336,47 (30,98)	ns
REM sleep	21,76 (10,49)	21,22 (2,93)	ns	48,48 (13,37)	44,56 (11,77)	ns	58,40 (13,52)	45,81 (18,30)	ns	58,60 (9,66)	58,25 (8,57)	ns
Total sleep time	200,88 (39,09)	220,06 (14,55)	ns	293,24 (28,17)	309,50 (31,35)	ns	339,50 (23,23)	325,22 (55,84)	ns	382,17 (25,82)	394,72 (30,52)	ns
Wakefulness episodes:												
Number	51,86 (13,26)	47,33 (14,69)	ns	48,29 (8,77)	54,33 (8,80)	ns	76,57 (13,44)	72,67 (17,33)	ns	77,14 (9,82)	61,83 (11,65)	ns
Mean duration	61,56 (15,64)	66,11 (17,61)	ns	48,25 (7,35)	41,22 (11,70)**	ns	29,02 (5,12)***	32,00 (7,56)***	ns	24,90 (3,55)***	31,09 (6,20)***	ns
SWS episodes:												
Number	86,57 (13,49)	84,83 (16,27)	ns	100,00 (12,19)	107,33 (16,77)**	ns	136,14 (21,15)***	137,33 (14,25)***	ns	146,14 (10,87)***	118,33 (15,55)***	<0,05
Mean duration	11,71 (1,34)	13,77 (2,67)	ns	14,32 (1,20)*	14,54 (1,16)	ns	12,18 (2,42)	11,96 (2,12)	ns	12,98 (1,18)	16,99 (2,49)	<0,01
REM sleep episodes:												
Number	13,86 (7,31)	13,33 (2,16)	ns	26,14 (7,01)	27,67 (7,00)	ns	32,14 (4,95)	27,50 (9,77)	ns	30,57 (4,86)	27,50 (3,73)	ns
Mean duration	9,39 (1,48)	9,17 (0,88)	ns	10,67 (1,33)	9,35 (0,59)	ns	10,51 (1,84)	9,47 (1,19)	ns	11,04 (1,09)	12,65 (2,16)	ns

Active Phase 19:00-07:00	CMS Day 4			CMS Day 5			CMS Day 6			CMS Day 7		
Sleep stages:	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*
Wakefulness	426,76 (24,27)	451,58 (62,47)	ns	361,57 (32,63)	391,81 (41,84)	ns	382,31 (47,93)	387,08 (35,62)	ns	394,50 (55,12)	432,14 (54,31)	ns
SWS	236,36 (23,93)	219,81 (50,88)	ns	292,81 (23,04)	274,19 (37,74)	ns	276,40 (35,92)	277,58 (27,22)	ns	248,60 (39,88)	227,83 (55,24)	ns
REM sleep	54,50 (10,89)	42,61 (15,93)	ns	63,52 (12,25)	50,69 (8,22)	ns	56,45 (14,41)	48,86 (9,33)	ns	47,36 (18,79)	34,14 (15,88)	ns
Total sleep time	290,86 (26,59)	262,42 (65,88)	ns	356,33 (33,61)	324,89 (43,48)	ns	332,86 (47,40)	326,44 (34,36)	ns	295,95 (56,52)	261,97 (70,18)	ns
Wakefulness episodes:												
Number	50,71 (8,10)	42,67 (17,26)	ns	66,14 (10,16)	58,67 (9,46)	ns	65,57 (14,01)	57,50 (14,07)	ns	59,86 (15,45)	43,17 (11,25)	ns
Mean duration	50,02 (10,18)	82,66 (60,28)	<0,01	33,07 (8,20)***	40,64 (10,38)**	ns	35,14 (9,69)**	43,57 (13,79)*	ns	41,97 (16,66)*	68,87 (23,41)	<0,05
SWS episodes:												
Number	109,14 (14,33)**	84,83 (38,88)	<0,05	132,43 (9,73)***	112,67 (19,64)**	ns	125,71 (17,87)***	107,33 (29,40)**	ns	116,43 (20,29)***	81,33 (24,70)	<0,01
Mean duration	12,71 (2,01)	16,38 (3,29)*	<0,01	12,93 (0,57)	14,40 (2,10)	ns	12,65 (1,38)	16,14 (4,25)*	<0,01	12,83 (2,88)	17,08 (3,50)**	<0,01
REM sleep episodes:												
Number	28,14 (6,26)	22,83 (10,36)	ns	32,43 (6,90)	29,67 (4,55)	ns	29,86 (7,78)	26,83 (6,65)	ns	24,29 (9,69)	19,17 (7,81)	ns
Mean duration	11,50 (0,69)	11,25 (1,08)	ns	11,53 (1,43)	9,89 (1,06)	ns	11,27 (2,01)	10,82 (1,28)	ns	11,02 (1,27)	10,29 (1,59)	ns

Table 3: Time (min) spent in wakefulness, slow wave sleep (SWS), rapid eye movement (REM) sleep, total sleep time, and number of episodes and mean episode duration (min) of wakefulness, SWS and REM sleep, during 12 h active phase during baseline and 7 days of chronic mild stress (CMS); in brief maternally separated rats (BMS, $n = 6$) and long maternally separated (LMS, $n = 7$) rats. All values represent mean (standard deviation). Significant within group differences (compared to baseline) are marked with * ($p<0,05$), ** ($p<0,01$) and *** (0,001), and significant between group differences are in column *p.*

Inactive Phase 07:00-19:00	Baseline			CMS Day 1			CMS Day 2			CMS Day 3		
Sleep stages:	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*
Wakefulness	176,93 (18,78)	177,56 (23,63)	ns	294,90 (32,84)	287,75 (38,24)	ns	254,17 (26,67)	240,86 (37,42)	ns	268,88 (43,23)	239,69 (16,80)	ns
SWS	432,55 (14,50)	429,47 (15,71)	ns	347,26 (21,91)	353,19 (37,80)	ns	382,57 (16,85)	389,22 (31,22)	ns	366,57 (26,94)	394,64 (16,05)	ns
REM sleep	99,00 (10,39)	95,47 (13,62)	ns	74,67 (15,30)	74,58 (12,76)	ns	81,10 (14,18)	86,97 (9,42)	ns	81,62 (19,83)	83,22 (10,06)	ns
Total sleep time	531,55 (19,33)	524,94 (19,92)	ns	421,93 (32,25)	427,78 (39,18)	ns	463,67 (26,85)	476,19 (37,56)	ns	448,19 (45,23)	477,86 (16,62)	ns
Wakefulness episodes:												
Number	61,43 (12,51)	60,33 (7,15)	ns	56,00 (15,21)	54,83 (13,73)	ns	67,43 (16,95)	64,33 (16,43)	ns	81,57 (13,20)	65,83 (16,42)	ns
Mean duration	14,57 (2,43)	15,63 (1,80)	ns	30,79 (5,69)	30,79 (7,87)	ns	21,83 (6,47)	21,76 (7,45)	ns	18,23 (3,32)	20,13 (4,66)	ns
SWS episodes:												
Number	156,57 (17,77)	146,67 (15,59)	ns	111,86 (11,65)	110,83 (16,13)	ns	141,57 (18,88)	126,50 (30,17)	ns	153,29 (13,43)	146,83 (23,91)	ns
Mean duration	16,39 (1,88)	17,57 (2,80)	ns	18,39 (2,25)	19,07 (3,77)	ns	16,24 (1,71)	19,22 (4,99)	ns	14,09 (1,40)	16,30 (2,81)	ns
REM sleep episodes:												
Number	47,43 (4,31)	43,83 (9,02)	ns	28,71 (3,86)	32,50 (6,44)	ns	35,43 (7,39)	33,17 (6,46)	ns	37,14 (9,53)	37,33 (8,38)	ns
Mean duration	12,13 (1,35)	13,08 (2,12)	ns	15,10 (1,48)	13,81 (2,62)	ns	13,60 (0,96)	15,92 (3,57)	ns	12,88 (1,30)	13,57 (2,44)	ns

Inactive Phase 07:00-19:00	CMS Day 4			CMS Day 5			CMS Day 6			CMS Day 7		
Sleep stages:	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*	LMS	BMS	*p*
Wakefulness	398,19 (30,93)	361,58 (16,78)	ns	264,29 (31,68)	227,19 (26,78)	ns	356,93 (62,45)	292,89 (31,85)	ns	263,17 (42,28)	241,78 (14,80)	ns
SWS	269,31 (22,89)	300,17 (12,55)	ns	375,69 (24,94)	400,17 (23,86)	ns	307,79 (51,15)	327,33 (44,16)	ns	385,45 (35,21)	400,11 (20,85)	ns
REM sleep	44,31 (14,20)	52,28 (8,82)	ns	73,69 (13,93)	85,42 (14,73)	ns	46,12 (11,28)	55,56 (6,94)	ns	64,95 (14,47)	67,81 (13,74)	ns
Total sleep time	313,62 (36,09)	352,44 (19,36)	ns	449,38 (34,68)	485,58 (30,67)	ns	353,90 (61,02)	382,89 (47,51)	ns	450,41 (45,06)	467,92 (16,08)	ns
Wakefulness episodes:												
Number	55,57 (13,14)	50,50 (9,87)	ns	76,43 (17,90)	66,50 (21,96)	ns	67,57 (9,54)	62,17 (14,20)	ns	74,71 (22,12)	68,00 (18,01)	ns
Mean duration	42,34 (7,39)	42,16 (7,36)	ns	19,72 (3,28)	18,94 (4,50)	ns	30,94 (7,05)	30,94 (10,47)	ns	22,12 (6,35)	20,62 (4,47)	ns
SWS episodes:												
Number	105,71 (8,16)	100,50 (21,12)	ns	153,57 (11,60)	137,17 (31,60)	ns	126,43 (20,49)	123,33 (33,50)	ns	144,29 (27,70)	127,83 (27,00)	ns
Mean duration	15,26 (1,77)	18,23 (3,44)	ns	14,50 (1,18)	18,59 (6,57)	ns	14,40 (0,93)	16,35 (3,63)	ns	16,44 (4,43)	19,64 (5,76)	ns
REM sleep episodes:												
Number	20,00 (7,09)	23,83 (5,38)	ns	32,14 (6,04)	36,33 (7,50)	ns	24,29 (5,94)	24,67 (5,61)	ns	33,00 (8,68)	33,33 (7,55)	ns
Mean duration	13,02 (1,75)	13,20 (2,39)	ns	13,32 (1,49)	13,98 (1,45)	ns	11,24 (1,61)	13,73 (3,14)	ns	11,63 (0,70)	12,06 (1,36)	ns

Table 4: Time (min) spent in wakefulness, slow wave sleep (SWS), rapid eye movement (REM) sleep, total sleep time, and number of episodes and mean episode duration (min) of wakefulness, SWS and REM sleep during 12 h inactive phase during baseline and 7 days of chronic mild stress CMS; in brief maternally separated (BMS, $n = 6$) and long maternally separated (LMS, $n = 7$) rats. All values represent mean (standard deviation). There were no significant within effects (compared to baseline) and no significant between group effects (column *p*).

SWS episodes:

There was an effect of CMS on number of SWS episodes and mean episode duration (all F's$_{(7,77)}$≤20,8; p's<0,001). Compared to baseline all animals had more episodes of SWS during active phase on each day of CMS and episodes were longer on day 1, 3, 4, 6 and 7 (all p's≤0,05). See *Table C* in Appendix. There was also an interaction effect of early life stress and CMS on both number of SWS episodes and mean episode duration (all F's$_{(7,77)}$≤3,7; p's<0,05). See *Table 3*. Compared to their own baseline, BMS group had more episodes of SWS on CMS day 1, 2, 3, 5 and 6 (all p's≤0,01). Mean episode duration was longer on CMS day 4, 6 and 7 (all p's≤0,05). LMS group had more episodes of SWS on CMS day 2, 3, 4, 5, 6 and 7 and longer mean episode duration on CMS day 1 compared to their own baseline (all p's≤0,01). Compared to BMS, LMS group showed more episodes of SWS but had shorter mean episode duration on CMS day 3, 4 and 7 (all p's≤0,05), in addition to shorter mean episode duration on CMS day 6 (all p's≤0,01).

REM sleep episodes:

There was an effect of CMS on number of REM sleep episodes and mean episode duration (all F's$_{(7,77)}$≤13,0; p's<0,001). Compared to baseline, all animals had more episodes of REM sleep on each day of CMS (all p's<0,001) and mean episode duration was longer on CMS day 3, 4, 5, 6 and 7 (all p's≤0,01). See *Table C* in appendix. There was no interaction effect of early life stress and CMS on either number of REM sleep episodes or for mean episode duration (F's$_{(7,77)}$≤0,6; p≤0,79), see *Table 3.*

Inactive phase:

Time spent in wakefulness and sleep

There was an effect of CMS on time spent in wakefulness, SWS and REM sleep, and total sleep time (all F's$_{(7,77)}$≤62,1; p's<0,001). During 12 h inactive phase on each day of

CMS, all animals spent more time in wakefulness, less time in both SWS and REM sleep and had less total sleep time compared to 12 h inactive phase during baseline (all p's <0,001). See *Table B* in appendix. There was no interaction effect of early life stress and CMS on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(7,77)}$≤1,8; p's≤0,55), see *Table 4*.

Wakefulness episodes:

There was an effect of CMS on number of wakefulness episodes and mean episode duration (all F's$_{(7,77)}$≤37,8; p's<0,001). Compared to baseline, all animals had more episodes of wakefulness on CMS day 3, 5 and 7 (all p's≤0,05). Mean wakefulness episode duration was longer on CMS day 1, 2, 4, 5, 6 and 7 (all p's≤0,05), and close to significant longer on CMS day 3, compared to baseline (p=0,051). See *Table C* in Appendix.

SWS episodes:

There was an effect of CMS on number of SWS episodes and mean episode duration (all F's$_{(7,77)\leq}$13,34; p's≤0,01). Compared to baseline, all animals had less episodes of SWS on CMS day 1, 2, 4, 6 and 7 (all p's≤0,05). Mean SWS episode duration was shorter on day 3 of CMS (p<0,05), and close to significant longer on CMS day 1, compared to baseline (p<0,051). See *Table C* in Appendix.

REM sleep episodes:

There was an effect of CMS on number of REM sleep episodes and mean episode duration (all F's$_{(7,77)}$≤29,3; p's<0,001). Compared to baseline, all animals had less episodes of REM sleep on each day of CMS (all p's<0,001). Mean REM sleep episode duration was longer on CMS day 1 and 2, compared to baseline (all p's≤0,01). See *Table C* in Appendix.

There was no interaction effect of early life stress and CMS on number of episodes or mean episode duration for either wakefulness, SWS or REM sleep (all $F's_{(7,77)} \leq 1{,}9$; $p's \leq 1{,}0$).

3.3 Effect of individual stressors during 1 week of CMS

The different stressors were analysed in 3 h intervals.

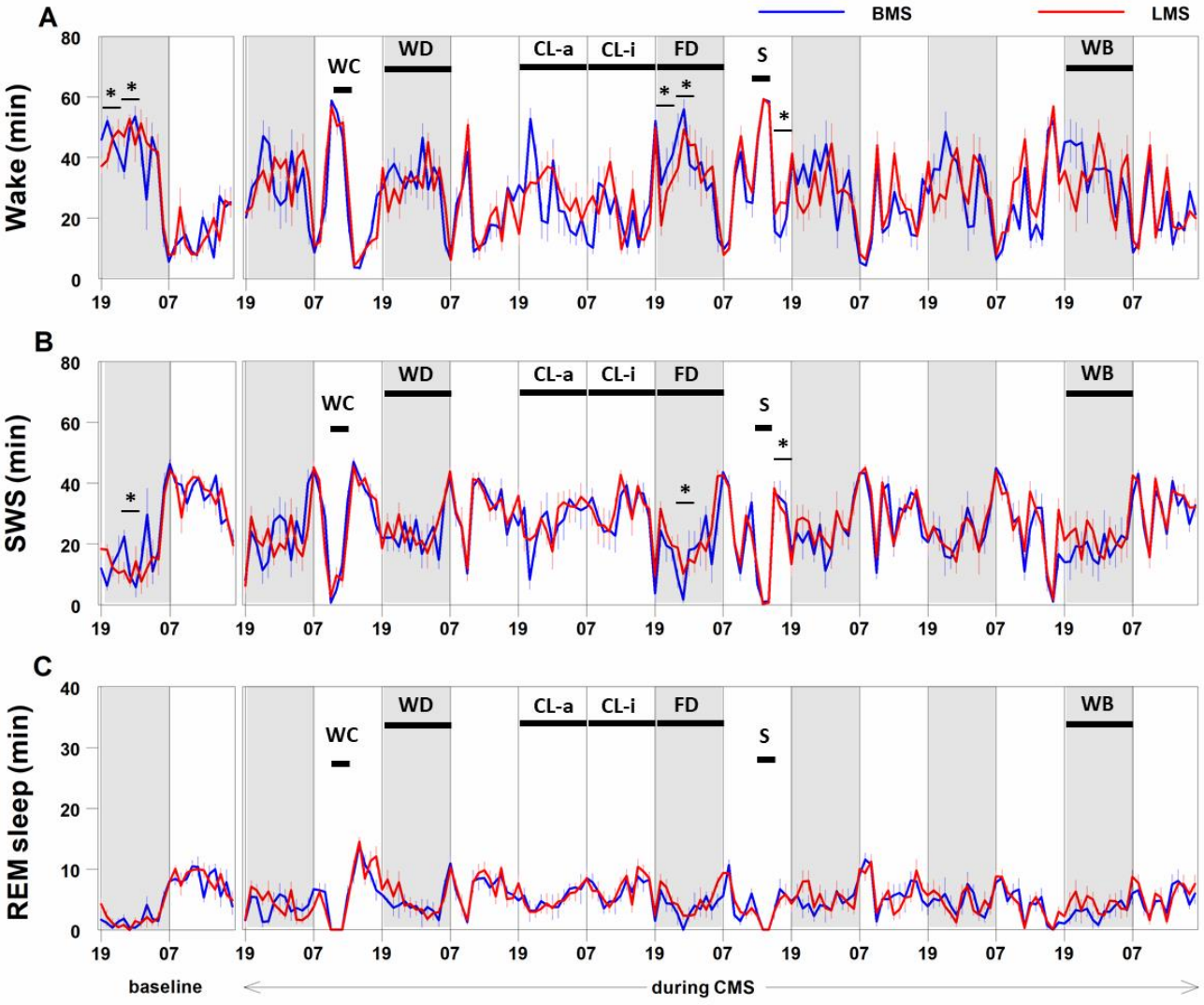

Figure 7: Time spent in wakefulness (panel A), slow wave sleep (SWS, panel B), and rapid eye movement (REM) sleep (panel C), in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7) during baseline and one week of exposure to chronic mild stress. Shaded areas represent dark period of the 12 h light/12 h dark schedule. Values are plotted in 1-h intervals, mean±SEM. Significant differences during baseline represent effect of early life stress. The different chronic mild stressors analysed are: water in cage (WC); water deprivation (WD); continuous light

during active phase (CL-a) and during inactive phase (CL-i); food deprivation (FD); social stress (S) and wet bedding (WB). * ($p<0,05$) between groups.

Water in cage

Analysis of the 3 h interval during exposure to water in cage (CMS day 1, 10.00-13.00, "WC" in *Figure 7*) showed an effect of stressor on time spent in wakefulness and SWS, and total sleep time (all F's$_{(1,11)}\leq$1299,3; p's<0,001). During exposure to water in cage compared to the corresponding time interval during baseline (10.00-13.00), all animals spent more time in wakefulness (159,21±13,02 min vs 29,18±7,63 min, p<0,001) (see *Figure 7*, panel A), less time in SWS (19,82±13,05 min vs 119,22 ±9,25 min, p<0,001) (see *Figure 7*, panel B), and had less total sleep time (19,82±13,05 min vs 148,50±9,12 min, p<0,001). There was no interaction effect of early life stress and water in cage on time spent in wakefulness and SWS, or total sleep time (all F's$_{(1,11)}\leq$0,6; p's≤0,74).

During exposure to water in cage none of the animals displayed REM sleep. See *Figure 7*, panel C.

Analysis of the 3 h interval after exposure to water in cage (CMS day 1, 13.00-16.00) showed no effect of stressor (all F's$_{(1,11)}\leq$2,9; p's≤0,17), or interaction effect of early life stress and water in cage on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(1,11)}\leq$0,9; p's≤0,90).

Water deprivation

During exposure to water deprivation (CMS day 2, 19.00-07.00, "WD" in *Figure 7*) there was an effect of stressor ($F_{(1,11)}$=86,1; p<0,001), and interaction effect of water deprivation and interval on time spent in wakefulness, time spent in REM sleep, and total

sleep time (all F's$_{(3,33)}$≤5,3; p's≤0,05), but no interaction effect for time spent in SWS ($F_{(3,33)}$=1,96; p=0,14). All animals spent less time in wakefulness and had more total sleep time during interval 1 (19.00-22.00), interval 2 (22.00-01.00), interval 3 (01.00-04.00) and interval 4 (04.00-07.00) compared to baseline (all p's≤0,05). Compared to baseline all animals spent more time in REM sleep during interval 1 (19.00-22.00) and interval 2 (22.00-01.00) (all p's<0,001), and close to significant (p=0,052) during interval 3 (01.00-04.00). See *Table D* in Appendix. There was no interaction effect of early life stress, water deprivation and interval on wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(3,33)}$≤2,0; p's≤0,34).

Continuous light exposure

Analysis of 3 h intervals during light exposure in active phase (CMS day 3, 19.00-07.00, "CL-a" in *Figure 7*) showed an effect of stressor on time spent in wakefulness, SWS and REM sleep, and total sleep time (all F's$_{(1,11)}$≤313,3; p's<0,001). The post hoc results reflect the same as the 12 h interval analysis of active phase (see *Table 3*) - less time in wakefulness, more time in SWS and REM sleep, and more total sleep time compared to baseline, and are hence not repeated here. There was no interaction effect of continuous light and interval, and no interaction effect of early life stress, continuous light and interval on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(3,33)}$≤1,7; p's≤0,85).

Analysis of the 3 h intervals during the following inactive phase (CMS day 3, 07.00-19.00, "CL-i" in Figure 3) showed an effect of stressor "continuous light" (all F's$_{(1,11)}$≤59,7; p's≤0,05), and an interaction effect of continuous light and interval on time spent in wakefulness, SWS and REM sleep, and total sleep time (all F's$_{(3,33)}$≤17,3; p's<0,001). During

interval 1 (07.00-10.00) and interval 2 (10.00-13.00) all animals spent more time in wakefulness, less time in SWS and had less total sleep time and spent less time in REM sleep during interval 2 compared to the corresponding time intervals in baseline (all p's≤0,01). During interval 4 (16.00-19.00) all animals spent less time in wakefulness, more time in SWS and REM sleep, and had more total sleep time compared to baseline (all p's≤0,05). See *Table E* in Appendix.

There was no interaction effect of early life stress, continuous light and interval on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(3,33)}$≤1,3; p's≤0,84).

Food deprivation

During exposure to food deprivation (CMS day 4, 19.00-07.00 "FD" in *Figure 7*) there was an effect of stressor on time spent in wakefulness, SWS and REM sleep, and total sleep time (all F's$_{(1,11)}$≤38,8; p's≤0,01). There was an interaction effect of early life stress, food deprivation and interval on time spent in wakefulness and SWS (all F's$_{(3,33)=}$3,1; p's<0,05), but not for REM sleep ($F_{(3,33)=}$1,7; p=0,18), and there was close to significant interaction effect for total sleep time ($F_{(3,33)=}$2,8; p=0,054).

See *Figure 8* for time spent in wakefulness during stressor "food deprivation" and during corresponding time interval in baseline. BMS group spent less time in wakefulness during interval 1 and interval 4 compared to baseline (both p's<0,05). LMS group spent less time in wakefulness during interval 1 and interval 2, both compared to their own baseline and compared to BMS group (all p's<0,05).

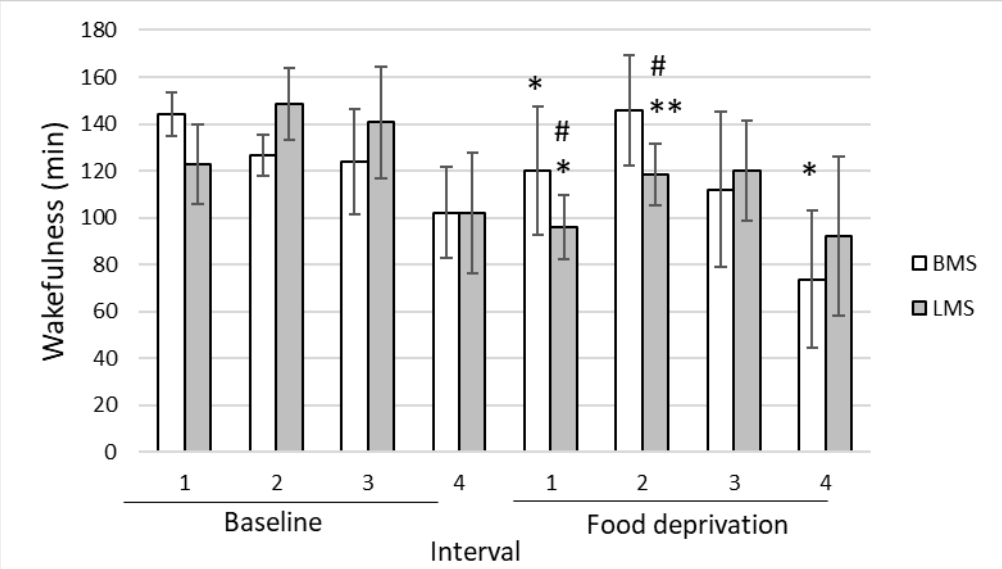

Figure 8: Time spent in wakefulness during 12 h active phase in baseline and during exposure to food deprivation, in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7). Error bars represent standard deviation. Significant differences are marked with * ($p<0,05$) compared to the corresponding time interval during baseline, and # ($p<0,01$) compared to BMS group.

See *Figure 9* for time spent in SWS during stressor "food deprivation" and during corresponding time interval in baseline. BMS group spent less time in SWS during interval 2 compared to baseline ($p<0,05$). LMS group spent more time in SWS during interval 2 compared to baseline and compared to BMS group (both p's$<0,05$).

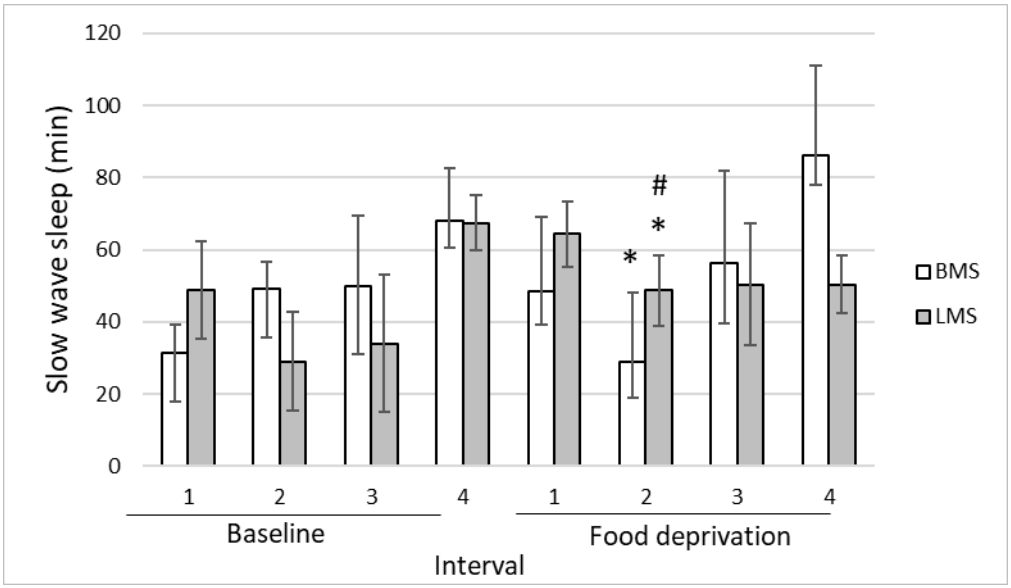

Figure 9: Time spent in slow wave sleep (SWS) during 12 h active phase in baseline and during exposure to food deprivation in brief maternally separated rats (BMS, n = 6) and long maternally separated rats (LMS, n = 7). Error bars represent standard deviation. Significant differences are marked with * ($p<0,05$) compared to the corresponding time interval during baseline, and # ($p<0,05$) compared to BMS group.

See *Figure 10* for total sleep time during stressor "food deprivation" and during corresponding time interval in baseline. BMS group had more total sleep time during interval 1 and interval 4 compared to baseline (both p's<0,05). LMS group had more total sleep time during interval 1 and interval 2 compared to baseline and compared to BMS group (all p's≤0,05).

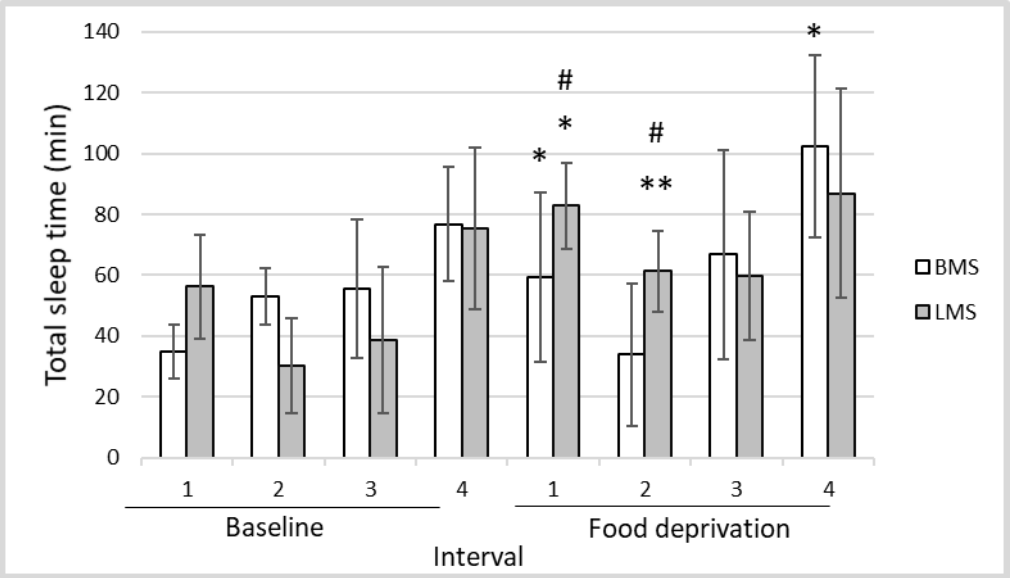

Figure 10: Total sleep time during 12 h active phase in baseline and during exposure to food deprivation in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7). Error bars represent standard deviation. Significant differences are marked with * ($p<0,05$) compared to the corresponding time interval during baseline, and # ($p<0,05$) compared to BMS group.

Social stress

Analysis of the 3 h interval during exposure to social stress (CMS day 4, 13.00-15.00, "S" in *Figure 7*) showed an effect of stressor on time spent in wakefulness, SWS and REM sleep, and total sleep time (all $F's_{(1,11)}\leq 987,6$; $p's\leq 0,001$). During social stress compared to the corresponding time interval during baseline, all animals spent more time in wakefulness (163,83±5,10 min vs 44,36±15,26 min; $p<0,001$) and less time in SWS (11,07±4,79 min vs 110,64±10,09 min; $p<0,001$), REM sleep (2,41±1,93 min vs 24,53±6,58 min; $p<0,001$), and had less total sleep time (13,49±5,27 min vs 135,17±15,40 min; $p<0,001$). There was no interaction effect of early life stress and social stress on time spent in wakefulness, SWS or REM sleep, or total sleep time (all $F's_{(1,11)}\leq 0,6$; $p's\leq 0,77$).

Analysis of the 3 h interval after exposure to social stress (CMS day 4, 16.00-19.00) showed an effect of stressor on time spent in SWS and total sleep time (all F's$_{(1,11)}$≤15,9; p's≤0,05), but not for time spent in wakefulness or REM sleep (all F's$_{(1,11)}$≤3,0; p's≤0,21). After social stress, all animals spent more time in SWS (102,82±20,03 min vs 81,38±11,29 min, p<0,01), and had more total sleep time (117,40±26,19 min vs 100,15±14,56 min, p<0,05) compared to the corresponding time interval during baseline.

There was an interaction effect of early life stress and social stress on time spent in wakefulness ($F_{(1,11)}$=6,2; p<0,05). See *Figure 11*. BMS group spent less time in wakefulness during the 3 h post social stress compared to the corresponding time interval during baseline (p<0,05). For LMS group there was no difference compared to baseline, however, LMS group spent more time in wakefulness after social stress, compared to BMS group (p<0,05).

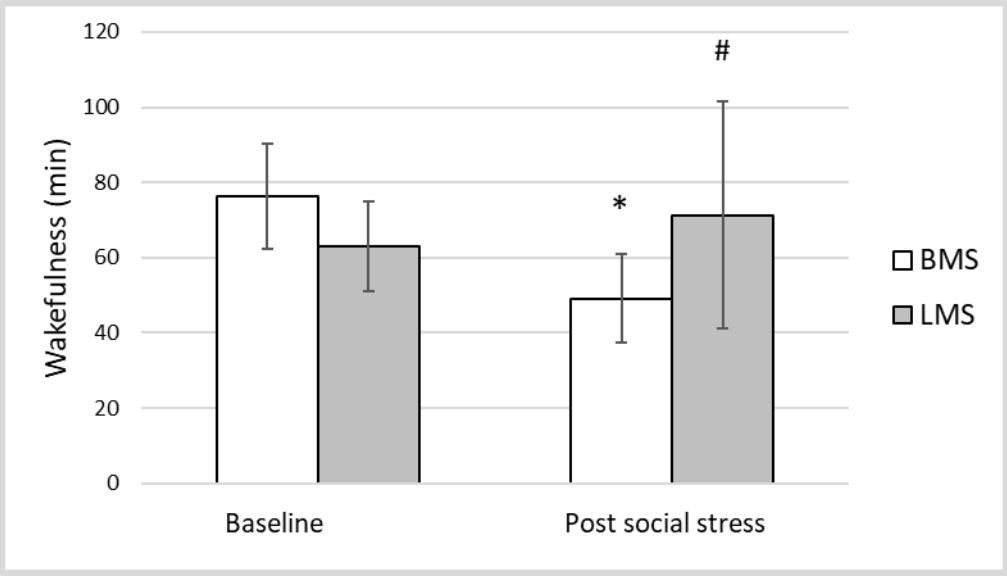

Figure 11: Time spent in wakefulness during 3 h baseline and 3 h post social stress in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7). Error bars represent standard deviation. Significant differences are marked with * (p<0,05) compared to the corresponding time interval during baseline, and # (p<0,05) compared to BMS group.

There was also an interaction effect of early life stress and social stress on time spent in SWS ($F_{(1,11)=}$7,3; p<0,05). See *Figure 12*. BMS group spent more time in SWS the following 3 h after social stress compared to the corresponding time interval during baseline (p<0,001). For LMS group there was no difference compared to baseline, however, LMS group spent less time in SWS after social stress compared to the BMS group (p<0,05).

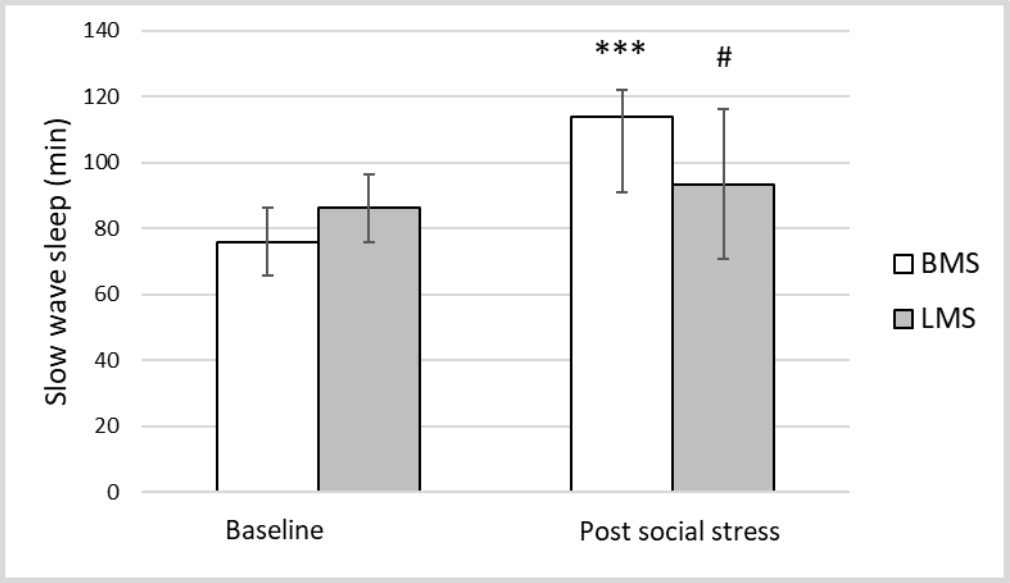

Figure 12: Time spent in slow wave sleep (SWS) during 3 h baseline and 3 h post social stress in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7). Error bars represent standard deviation. Significant differences are marked with *** (p<0,001) compared to the corresponding time interval during baseline, and # (p<0,05) compared to BMS group.

There was an interaction effect of early life stress and social stress on total sleep time ($F_{(1,11)=}$6,2; p<0,05). See *Figure 13*. BMS group had more total sleep time the following 3 h after social stress compared to the corresponding time interval during baseline (p<0,01). For LMS group there was no difference compared to baseline, however, LMS group spent less time in total sleep after social stress compared to BMS group (p<0,05).

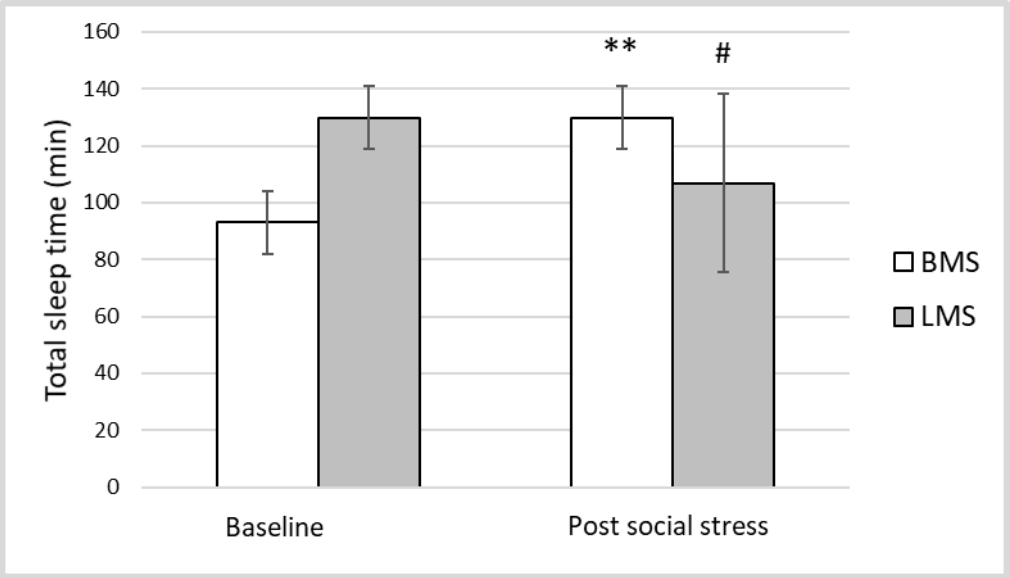

Figure 13: Total sleep time during 3 h baseline and 3 h post social stress in brief maternally separated rats (BMS, *n* = 6) and long maternally separated rats (LMS, *n* = 7). Error bars represent standard deviation. Significant differences are marked with ** ($p<0,01$) compared to the corresponding time interval during baseline, and # ($p<0,05$) compared to BMS group.

There was no significant interaction effect of early life stress and social stress on time spent in REM sleep ($F_{(1,11)=}1,3$; $p=0,27$).

Wet bedding

Analysis of 3 h intervals during exposure to wet bedding (CMS day 7, 19.00-07.00, "WB" in *Figure 7*) showed an effect of stressor on time spent in wakefulness, SWS and REM sleep, and total sleep time (all F's$_{(1,11)}\leq 23,3$; p's$\leq 0,05$). The post hoc results reflect the same as the 12 h interval analysis (*see Table 3*) - less time spent in wakefulness, more time in SWS and REM sleep, and more total sleep time compared to baseline, and are hence not repeated here. There was no interaction effect of wet bedding and interval and no interaction effect of early life stress, wet bedding and interval on time spent in wakefulness, SWS or REM sleep, or total sleep time (all F's$_{(3,33)}\leq 1,5$; p's$\leq 0,84$).

4.0 Discussion

4.1 Effect of early life stress on sleep and wakefulness

The first aim of the study was to investigate the effects of early life stress on sleep and wakefulness during both active and inactive phase, in LMS compared to BMS animals.

12 h active phase

The hypothesis for 12 h active phase was that LMS offspring would display similar time spent in wakefulness, SWS and REM sleep and similar total sleep time, as well as similar number and duration of wakefulness, SWS and REM sleep episodes compared to BMS offspring. The hypothesis was confirmed as results from 12 h interval analysis showed that there was no effect of early life stress on time spent in wakefulness, SWS or REM sleep or for total sleep time during 12 h active phase, or for number and duration of wakefulness, SWS and REM sleep episodes. Previous studies investigating effects of exposure to early life stress on sleep and wakefulness during the rats' active phase are scarce, as earlier studies mostly focus on sleep and wakefulness during the rats' inactive phase. One study from Tiba and colleagues found that there were no differences in time spent in SWS, time spent in REM sleep and number of REM sleep episodes during 12 h active phase between LMS (180 min daily) and BMS offspring (15 min) (Tiba et al., 2004), which supports my findings. In addition, Tiba and colleagues (2004) found no differences in the same sleep-parameters in LMS offspring compared to NH offspring. They found no differences in number of wakefulness episodes during active phase, which is consistent with my study, however, they did not investigate time spent in wakefulness or number of SWS episodes (Tiba et al., 2004).

In two studies using a more severe form of LMS, (6 h total daily separation, 2 * 3 h sessions) there were also no differences in time spent in wakefulness, SWS and REM, and total sleep time during 12 h active phase compared to handled animals (separated but immediately returned to their home cages) (Feng et al., 2012; Feng et al., 2007).

In the present study an 3 h interval analysis was performed for time spent in different sleep stages during active phase. The results showed an interaction effect of early life stress and interval on time spent in wakefulness and SWS, and total sleep time. During the first interval of active phase, LMS offspring spent less time in wakefulness, and had more total sleep time compared to BMS offspring, but for the second interval this was reversed as LMS offspring spent more time in wakefulness and less time in SWS and had less total sleep time compared to BMS offspring. These differences were mostly due to some variation between the intervals within the BMS offspring (see *Figure 7*). Despite these significant differences between LMS and BMS offspring during the two first intervals, there were no overall differences during the 12 h active phase on time spent in wakefulness, SWS or REM sleep, or total sleep time. Furthermore, all animals spent most of their active phase in wakefulness, which is as expected for rats, as they are nocturnal animals. Only one previous study has investigated 2 h intervals during active phase, and reported no differences in time spent in wakefulness, SWS or REM sleep, or total sleep time between LMS and BMS offspring (Tiba et al., 2004).

These findings indicate that there is need for further studies investigating the effects of early life conditions on sleep and wakefulness, as well as for number and duration of sleep and wakefulness episodes during active phase. Future studies should also compare different early life conditions such as LMS, BMS and NH in the same study.

12 h inactive phase

The hypothesis for 12 hours inactive phase was that LMS offspring were expected to display similar time spent in wakefulness, SWS and REM sleep, and total sleep time, as well as similar number and duration of wakefulness, SWS and REM sleep episodes compared to BMS offspring. The hypothesis was confirmed as results from 12 h interval analysis showed that there was no effect of early life stress on time spent in wakefulness, SWS or REM sleep, or for total sleep time, or for number and duration of wakefulness, SWS and REM sleep episodes during 12 h inactive phase. A previous study from Mrdalj and colleagues using the same animals and protocol showed that there were no significant differences on time spent in wakefulness or REM sleep, or for total sleep time, as well as no differences on number and duration of episodes of wakefulness and REM sleep between LMS and BMS offspring during 12 h inactive phase (Mrdalj et al., 2013). Hence, the results for inactive phase in the present study are confirming these findings in the same animals. However, Mrdalj and colleagues did find a difference in the quality of SWS between the two early life condition groups, with LMS offspring showing less SWS1 (light sleep), and more SWS2 (deep sleep) compared to BMS offspring during inactive phase (Mrdalj et al., 2013). In my analyses I have not differentiated between SWS1 and SWS2 and therefore not been able to investigate this difference in quality of SWS between the two groups. However, when looking at the total amount of SWS reported by Mrdalj et al., the results seem to reflect the present study for both LMS offspring (447,13 min vs 432,55 min) and BMS offspring (432,63 min vs 429,47 min).

Also, the overall agreement between the sleep scorings made in the present study and sleep scorings in the 2013 study was very high (total agreement of 93,95%±1,04%), and kappa value for SWS was 0,89±0,02.

There are several other studies investigating effects of exposure to early life stress on sleep and wakefulness during the rats' inactive phase. One study from Tiba and colleagues looked at 10 h of inactive phase and found that there were no differences on time spent in SWS or number of wakefulness episodes in LMS compared to BMS animals, which reflect the results in the present study. In addition, Tiba and colleagues found no differences in the same parameters in LMS offspring compared to NH offspring (Tiba et al., 2004). They did not investigate time spent in wakefulness or number of SWS episodes. However, in contrast to the present study they did find that LMS offspring displayed more time in REM sleep and had more REM sleep episodes during inactive phase compared to BMS offspring (Tiba et al., 2004). In a study using a more severe form of LMS (6 h daily separation, 2*3 h sessions) it was found that LMS offspring spent more time in wakefulness and had less total sleep time during 10 h inactive phase compared to handled offspring (separated, but immediately returned to their home cages). For time spent in SWS and REM sleep there were no significant differences between the two groups (Feng et al., 2007). Another study from the same authors using the same protocol found that REM sleep was significantly reduced, whereas time spent in wakefulness was significantly increased in the LMS offspring compared to the handled offspring during 10 h inactive phase (Feng et al., 2012). In a study using a mild approach to early life stress called cross-fostering (litters are changed between mothers) at PND 12 found that cross-fostered rats spent less time in wakefulness, more time in SWS and REM sleep, and had an increased number of REM sleep episodes during 12 h

inactive phase compared to NH rats (Santangeli et al., 2016). Overall, the results from previous studies indicate that differences in wakefulness and sleep between offspring from early life conditions may be a consequence of different protocols use, as well as the severity of stress exposure.

A 3 h interval analysis was also performed for time spent in different sleep stages during inactive phase. The results showed no interaction effect of early life stress and interval. This is consistent with findings from Tiba et al., on 2 h intervals during inactive phase (2004). Findings across the present study and previous studies reflect that the effects of early life stress on sleep are inconsistent and urge for more investigation. This may be due to differences in protocols used in the different studies. Future studies on early life stress on sleep should therefore include different early life conditions. Furthermore, future studies should include both active and inactive phase when investigating the effects of early life stress on sleep, as well as the recording period should also be done for a longer period of time.

4.2 Effect of 1 week of CMS on sleep and wakefulness

The second aim of the study was to investigate how one week of chronic mild stress in adulthood would affect sleep and wakefulness during stress exposure, in both active and inactive phase, in rats from different early life conditions (LMS, BMS).

12 h active phase during 1 week of CMS

The hypothesis was that across 7 days of CMS during active phase, both LMS and BMS offspring were expected to show a decrease of time spent in wakefulness and increased

total sleep time; and LMS would show less time spent wakefulness and more total sleep time, compared to BMS offspring.

The results showed an effect of CMS on time spent in wakefulness, SWS and REM sleep and total sleep time for all animals. During active phase, on each day of CMS, both LMS and BMS offspring spent less time in wakefulness and spent more time in SWS and REM sleep and had more total sleep time compared to 12 h active phase during baseline. There were no differences between LMS and BMS offspring. Hence, the hypothesis was confirmed as both LMS and BMS offspring spent more time in wakefulness and had more total sleep time across 7 days of CMS. There were no significant differences between the two groups on time spent in wakefulness, or for total sleep time, hence, the hypotheses that LMS offspring would show a stronger decrease in wakefulness and stronger increase in total sleep time compared to BMS offspring was not confirmed.

However, the results showed an effect of CMS on number and duration of wakefulness, SWS and REM sleep episodes for all animals during active phase. Overall, all animals had more episodes of wakefulness and REM sleep during some of the CMS days, and more episodes of SWS on each day of CMS compared to baseline. The duration of each episode of wakefulness was shorter, and the duration of each SWS and REM sleep episode was longer during CMS compared to baseline. For mean episode duration of wakefulness these effects were larger in LMS offspring compared to BMS offspring especially during CMS day 4 (food deprivation during active phase) and CMS day 7 (wet bedding during active phase), as well as mean episode duration of SWS was shorter during some of the CMS days compared to BMS (see *Table 4*).

These findings are interesting as there exist no other studies which have investigated how sleep and wakefulness in LMS and BMS offspring are affected during CMS. These findings shows that CMS results in a disruption of the normal distribution of wakefulness and sleep in both groups, with more fragmented wakefulness during active phase. More importantly, the results show a stronger effect of CMS in LMS offspring, as LMS offspring have longer mean episode duration of wakefulness and shorter mean episode duration of SWS, compared to BMS offspring. The study from Mrdalj et al (2013), did not investigate sleep and wakefulness in LMS and BMS offspring during active phase after exposure to CMS. As there exists no previous studies on sleep and wakefulness in LMS and BMS offspring during active phase during or after exposure to CMS, my findings contribute with interesting, new data. This also emphasizes the need for further studies investigating the effects of CMS on sleep and wakefulness in animals exposed to early life stress.

12 h inactive phase during 1 week of CMS

The hypothesis was that across 7 days of CMS during inactive phase, both LMS and BMS offspring would show increased time spent in wakefulness and less total sleep time; and LMS offspring would show less total sleep time as well as increased number of REM sleep episodes compared to BMS offspring.

The results showed an effect of CMS on time spent in wakefulness, SWS and REM sleep, and for total sleep time for all animals. During inactive phase, on each day of CMS, both LMS and BMS offspring spent more time in wakefulness, and less time in SWS and REM sleep, and had less total sleep time compared to 12 h inactive phase during baseline. Hence, the hypothesis was confirmed. Exposure to different chronic mild stressors for one

week resulted in more wakefulness and less sleep during inactive phase, when animals are normally expected to rest. Exposure to different stressors occurred during both active and inactive phase, and some of the stressors were presented only during the rats' inactive phase. Furthermore, all animals had more and longer episodes of wakefulness, as well as less episodes of SWS and REM sleep during inactive phase on most days of CMS. These results suggest that sleep was much more fragmented during exposure to CMS in both LMS and BMS offspring. In the study from Mrdalj and colleagues (2013) using the same animals and CMS protocol, it was found that after exposure to 4-weeks of CMS (two days after ended exposure to CMS), sleep and wakefulness were affected in both groups. Both LMS and BMS offspring showed less total sleep time and more time spent in wakefulness. The present study focused on effects on sleep and wakefulness during exposure to one week of CMS and confirmed these findings on more time spent in wakefulness and less total sleep time in both LMS and BMS offspring. Furthermore, the findings show an overall more fragmented sleep in inactive phase across one week during exposure to CMS.

Mrdalj et al also found that LMS offspring had more total sleep time and more REM sleep episodes compared to BMS offspring after ended CMS exposure (2013). Therefore in the present study during exposure to CMS, it was expected that LMS offspring would show less total sleep time and an increased number of REM sleep episodes compared to BMS offspring. However, this was not confirmed. In fact, the analyses did not reveal any differences between LMS and BMS offspring during CMS exposure for any of the parameters of sleep and wakefulness (time spent in wakefulness and sleep, and for number and duration of wakefulness and sleep episodes). The study from Mrdalj et al., looked at sleep and

wakefulness in LMS and BMS offspring after exposure to CMS. However, only inactive phase was investigated.

No other studies have examined sleep and wakefulness in LMS and BMS offspring during exposure to CMS or after exposure to CMS. A general effect of CMS on sleep and wakefulness was described in a study from Grønli and colleagues (2004). The study used the same CMS protocol as in the present study and found that after 4 weeks of CMS the animals spent less time in wakefulness and had more wakefulness episodes and spent less time in SWS-2 (deep sleep) but more time in SWS-1 (light sleep) and REM sleep. This study was not designed to investigate effects of CMS in maternally separated rats. The findings are therefore not directly comparable with my results, but they give an indication of how CMS affects sleep and wakefulness, and that the sleep-wake cycle is disrupted following CMS. The study only looked at 8 h of inactive phase and did not investigate sleep and wakefulness in the active phase. The results from the present study indicate strongly that the effects of CMS on sleep and wakefulness during active phase should be more investigated, particularly in LMS and BMS offspring. Overall, there is a need for further studies on how sleep and wakefulness are affected during and after exposure to CMS in LMS and BMS offspring.

4.3 Effect of individual stressors on sleep and wakefulness

One specific aim of the study was to investigate if different individual mild stressors during CMS would affect sleep and wakefulness differently in LMS and BMS offspring. It was expected that individual stressors would affect sleep and wakefulness differently in LMS and BMS offspring. The differential effect was expected in particular for stressors: water deprivation, continuous light, food deprivation, social stress, and wet bedding.

The analyses revealed an interaction effect between early life stress, stressor and interval for stressors “food deprivation” and “social stress” but not for stressors “water in cage”, “water deprivation”, “continuous light” or “wet bedding”.

Food deprivation was previously found to provoke a stronger reduction in body temperature in LMS compared to BMS (Mrdalj 2014). In the present study, it was found that food deprivation reduced time spent in wakefulness and increased total sleep time in both LMS and BMS offspring, during the first part of the active phase. The results also indicate that food deprivation had a stronger effect on LMS offspring during the first part of active phase since LMS offspring spent less time in wakefulness and more time in SWS and had more total sleep time compared to BMS offspring. Moreover, the 12 h interval analysis showed more episodes of SWS in LMS offspring compared to BMS during the entire active phase. No other studies have investigated effect of food deprivation in LMS or BMS offspring, and hence the results from the present study suggest further investigation is needed, as well as comparison between different early life conditions.

During exposure to social stress, all animals spent more time in wakefulness, and had less SWS and REM sleep, and had less total sleep time compared to the corresponding 3 h interval during baseline. In fact, the animals were awake during almost the entire stress exposure and were on average asleep for only 14 minutes (compared to 135 min during corresponding time interval in baseline). This is not a surprising finding as the animals were facing a social challenge by meeting another unfamiliar male rat (paired caging). To prevent development of dominant behaviour rats alternated between being the intruder and the resident. However, meeting between two previously unfamiliar male rats will most likely result in a social conflict, and hence increasing arousal (Yamada-Haga, 2002). A study from

Mrdalj and colleagues (2014) using the same protocol and the same animals as in the present study showed that social stress (paired caging) during inactive phase resulted in a tendency for lower body temperature in LMS offspring compared to BMS offspring. Based on this finding it was expected in the present study to find a stronger effect of social stress on time spent in wakefulness and sleep in LMS compared to BMS offspring during stress exposure, but there were no significant differences between the two early life conditions. After social stress, although all animals reduced time in wakefulness and increased time in sleep, LMS offspring spent more time in wakefulness, had less time in SWS and less total sleep time, compared to BMS offspring. Hence, the stronger effect of social stress in LMS offspring was evident after exposure to social stress, when animals were left undisturbed in their home cages. Increased stress reactivity in LMS compared to BMS offspring has been demonstrated in previous studies measuring activity in the HPA axis (Mrdalj et al., 2014; Plotsky & Meaney, 1993). Although present study did not investigate stress reactivity measured by level of stress hormones, it is possible that the increase in wakefulness after social stress may reflect increased stress reactivity in LMS offspring.

Exposure to a more severe form of social challenge (social defeat, where the animal is physically defeated by a more aggressive male) has been shown to accelerate the build-up of sleep pressure (SWA) after stress exposure (Meerlo et al., 1997). This is not directly comparable to the social stress in the present study; however, it may indicate that sleep quality and sleep pressure after exposure to social stress should be investigated in LMS and BMS offspring. Moreover, it has been shown that LMS offspring have slower reduction of sleep pressure compared to BMS, after exposure to 4 weeks of CMS (Mrdalj 2013). There exist no other studies investigating sleep and wakefulness during and after exposure to social stress in

LMS and BMS offspring. As indicated by the findings in the present study this should be further investigated including offspring from different early life conditions.

The impact of other mild stressors, “water in cage”, “water deprivation”, “continuous light” and “wet bedding” on sleep and wakefulness in LMS and BMS offspring, was also investigated. In the previous study on the same animals it was found that LMS compared to BMS offspring responded with stronger reduction in body temperature during exposure to water deprivation and wet bedding, and there was a tendency for stronger hypothermia in LMS offspring during exposure to continuous light (Mrdalj et al., 2014). Based on this it was expected that the present study would reveal differences in sleep and wakefulness between LMS and BMS offspring. However, the analysis of individual stressors did not show different impact of these stressors in LMS compared to BMS offspring. During exposure to water deprivation, continuous light and wet bedding, offspring from both early life conditions spent less time in wakefulness and had more total sleep time compared to baseline. In addition, during exposure to continuous light during active phase, offspring from both early life condition groups spent more time in SWS and REM sleep compared to baseline. Continuous light during active phase has previously been shown to affect sleep, with an increased total sleep time as well as increased time in SWS and REM sleep, and reduced wakefulness (Stenvers et al., 2016). These findings reflect the results in the present study. Stenvers and colleagues (2016) speculate that the effect of light exposure during active phase on sleep may be mediated by decreased levels of hormone melatonin as melatonin release is normally inhibited by light in both nocturnal and diurnal animals (Pévet, 2003). The present study did

not measure melatonin levels, however a previous study on rats found reduced melatonin levels in LMS offspring (2*3 h daily) compared to handled offspring (Feng et al., 2012).

During exposure to water in cage all animals spent more time in wakefulness and less time in SWS and had less total sleep time compared to the same time interval during baseline. This is not surprising given that 3 cm of water in the cage makes it difficult for the rats to fall comfortably asleep, hence time spent in wakefulness will increase as time spent in sleep will decrease.

Findings from the present study clearly indicate that CMS affects sleep and wakefulness in both LMS and BMS offspring showing a disturbance in the normal distribution of sleep and wakefulness during both active and inactive phases. Moreover, some of the mild stressors induced a stronger effect in LMS compared to BMS offspring, suggesting a difference in stress responsivity as a consequence of different early life conditions. Yet, it is not possible to draw any strong conclusions based only on these findings. More studies are needed to further investigate sleep and wakefulness in offspring form different early life conditions during exposure to CMS, and especially during exposure to different mild stressors, particularly food deprivation and social stress.

4.4 Strengths and limitations of the study

Sample size

One limitation of this experiment is the small sample size. Data on sleep and wakefulness were collected from 16 male Wistar-rats (8 LMS and 8 BMS), and Only 13 animals were used for analysis, as one animal died due to postoperative complications, while two animals were excluded from further analysis due to artifacts in the EEG signals. This

small sample size provides small statistical power, and the results can therefore not be generalized. Future studies investigating sleep should therefore include a larger sample size. However, even with small sample size we found significant differences in sleep and wakefulness between LMS and BMS offspring during exposure to CMS in adulthood. NH condition was not included in the present study due to the limited number of transmitters available in the lab. Future studies should also investigate how exposure to CMS affects sleep and wakefulness in NH offspring and offspring from other early life conditions.

SWS characterization

Mrdalj and colleagues (2013) found a difference in quality of SWS in LMS and BMS offspring as LMS offspring displayed less SWS1 (light sleep), and more SWS2 (deep sleep) during inactive phase, compared to BMS offspring. In the present study it was not differentiated between SWS1 and SWS2. Instead, both light sleep and deep sleep were scored as SWS. Therefore, the present study could not test the hypothesis or replicate the results from Mrdalj et al (2013) considering the quality of SWS in the offspring. While this is a drawback of the present study, the merging of SWS1 and SWS2 made the manual scoring procedure less time-consuming and hence, analysis of a large amount of EEG data was possible (24 h baseline and 7 days of continuous EEG recording during CMS, for each animal). This also enabled further analyses using the SLEEP-report script in MATLAB to extract the information about time spent in different sleep stages, as well as number and duration of sleep and wakefulness episodes, from such a large amount of data. The quality of SWS may also be addressed by performing EEG power analyses and should be applied in future studies on sleep in offspring from different early life conditions.

Surgical procedure

In the present study an invasive surgical procedure involving implantation of transmitters was performed in order to obtain EEG and EMG data. Surgery can affect sleep quality (Gögenur, 2010), however, previous studies using similar surgical procedures have found that one week of postoperative recovery is enough for both the amount of SWS and REM sleep to stabilize, both during the active and inactive phase (Tang et al., 2007). A minimum of two weeks postoperative recovery was allowed after surgery in the present study. As all animals from both groups (LMS, BMS) were exposed to the same procedures, there is no reason to believe that the differences between the two groups are due to surgery, but an effect of different early life conditions, i.e. LMS and BMS. Still, surgical procedure may affect LMS and BMS offspring differently given the differences in the stress regulating mechanisms in the two groups (Plotsky & Meaney, 1993), and hence influence the results.

Gender perspectives

To control for prenatal conditions in the offspring used in this study, female Wistar-rats were mated with male Wistar-rats in the animal facility at Department of Biological and Medical Psychology, University of Bergen. However, only male offspring were used for this study. The majority of preclinical studies are performed on male animals, which is a limitation in the present study as well. Early life stress is associated with an increased risk of developing anxiety- and depression-related behaviour in both humans and animals. However, women are more likely than men to develop anxiety-related disorders during life (McLean et al., 2011), and depression is more prevalent in women than in men (Albert, 2015). Early life

stress has also been found to affect the regulation of the HPA axis, with more negative outcomes in female subjects than in male subjects (Weiss et al., 1999). Animal studies have also shown that female rat offspring are more vulnerable to develop anxiety- (Honeycutt et al., 2020) and depression-like behaviour after exposure to early life stress (Mourlon et al., 2010). Later life stress in adulthood, such as exposure to chronic variable stress in adulthood have been shown to alter HPA axis activity in female rats compared to male rats (Renard et al., 2007, 2010). These studies emphasizes that gender should be considered as a biological variable when investigating the effects of early- and later life stress. Furthermore, only one study has investigated sleep in female rats from different early life conditions upon exposure to an acute stressor in adulthood. In brief, it was found that 1 h of cold stress increased time spent in REM sleep in LMS offspring compared to BMS and NH offspring (Tiba et al., 2008). Hence, future studies should include female rats when investigating the effects of early life stress on sleep and upon exposure to chronic stress in adulthood.

Summary and conclusion

The present study investigated sleep and wakefulness in rats exposed to long-maternal separation and brief-maternal separation and how sleep and wakefulness were affected during exposure to chronic mild stress in adulthood. As a consequence of different early life conditions the results showed no differences in sleep and wakefulness in either active phase or inactive phase as LMS and BMS offspring displayed similar time in wakefulness, SWS and REM sleep and had similar total sleep time.

This study is the first to investigate sleep and wakefulness during both active and inactive phase during exposure to CMS in offspring from different early life conditions (LMS

and BMS). One week of CMS exposure affected sleep and wakefulness similarly in all LMS and BMS offspring. During active phase across all CMS days, both LMS and BMS offspring displayed less time in wakefulness and spent more time in SWS and REM sleep and had more total sleep time. During inactive phase across all CMS days, both LMS and BMS offspring displayed more time in wakefulness and spent less time in SWS and REM sleep and had less total sleep time. These results show that exposure to chronic mild stressors affects distribution of sleep and wakefulness during both active and inactive phase.

The study also aimed to investigate effect of individual mild stressors on sleep and wakefulness in LMS and BMS offspring, and the results showed that the stressors "food deprivation" and "social stress" induced a stronger effect in LMS compared to BMS offspring. During food deprivation LMS offspring showed less wakefulness and more total sleep time compared to BMS offspring. After exposure to social stress the LMS offspring spent more time in wakefulness, less time in SWS and had less total sleep time compared to BMS offspring.

Overall, the findings in the present study add to the literature on the consequences of early life stress in combination with chronic mild stress in adulthood on sleep and wakefulness, both during active phase, and inactive phase. These results also suggest a difference in stress reactivity as a consequence of different early life conditions. More studies are needed to further investigate sleep and wakefulness in offspring form different early life conditions during exposure to CMS, and especially during exposure to different mild stressors, particularly food deprivation and social stress. The present study included only LMS and BMS male offspring. Future studies should include both male and female offspring from different early life conditions.

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Appendix

	Interval 1	**Interval 2**	**Interval 3**	**Interval 4**
Active Phase	**19:00-22:00**	**22:00-01:00**	**01:00-04:00**	**04:00-07:00**
Wakefulness	132,69 (17,31)	138,49 (16,75)	132,87 (24,0)	102,03 (22,07)***, ###, &&&
SWS	40,77 (13,98)	38,38 (15,06)	41,36 (20,21)	67,71 (19,19) ***, ###, &&&
REM sleep	5,62 (4,80)	2,36 (2,50)	5,17 (4,75)#	8,37 (4,64)***, ###, &
Total sleep time	46,38 (17,45)	40,74 (17,13)	46,53 (24,11)	76,08 (22,43) ***, ###, &&&
Inactive Phase	**07:00-10:00**	**10:00-13:00**	**13:00-16:00**	**16:00-19:00**
Wakefulness	34,60 (18,92)	29,18 (7,63)	44,36 (15,26)#	69,08 (14,10)***, ###, &&&
SWS	119,88 (15,03)	119,22 (9,25)	110,64 (10,09)	81,38 (11,29)***, ###, &&&
REM sleep	24,79 (6,10)	29,28 (4,61)	24,53 (6,58)	18,77 (5,26)##
Total sleep time	144,68 (18,23)	148,50 (9,12)	135,17 (15,4)#	100,15 (14,56)***, ###, &&&

Table A. Effect of interval on time spent in wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and total sleep time during 12 h active phase and 12 h inactive phase during baseline (*n*=13). All values are in minutes. Results are given as mean and standard deviation (±SD). Significant differences are marked with * = compared to interval 1, # = compared to interval 2, & = compared to interval 3. #, & ($p<0,05$), ## ($p<0,01$) and ***, ###, &&& ($p<0,001$).

Active Phase	**Baseline**	**CMS Day 1**	**CMS Day 2**	**CMS Day 3**	**CMS Day 4**	**CMS Day 5**	**CMS Day 6**	**CMS Day 7**
Wakefulness	506,08 (28,99)	387,09 (31,04)*	382,41 (36,36)*	329,96 (28,14)*	438,21 (45,68)*	375,53 (38,83)*	384,51 (41,03)*	411,87 (55,94)*
SWS	188,22 (24,54)	254,08 (23,66)*	280,32 (28,30)*	329,53 (27,59)*	228,72 (37,93)*	284,22 (30,86)*	276,95 (30,89)*	239,01 (46,72)*
REM sleep	21,51 (7,66)	46,67 (12,30)*	52,59 (16,55)*	58,44 (8,79)*	49,01 (14,25)*	57,60 (12,14)*	52,95 (12,47)*	41,26 (18,13)*
Total sleep time	209,73 (30,84)	300,74 (29,62)*	332,91 (40,30)*	387,96 (27,64)*	277,73 (48,78)*	341,82 (40,23)*	329,90 (40,33)*	280,27 (62,93)*
Inactive Phase								
Wakefulness	177,22 (20,22)	291,60 (34,10)*	248,03 (31,41)*	255,41 (35,80)*	381,30 (30,93)*	247,17 (34,22)*	327,37 (58,96)*	253,30 (33,29)*
SWS	431,13 (14,51)	350,00 (29,07)*	385,64 (23,66)*	379,53 (26,12)*	283,55 (24,17)*	386,99 (26,64)*	316,81 (47,16)*	392,22 (29,31)*
REM sleep	97,37 (11,60)	74,63 (13,60)*	83,81 (12,12)*	82,36 (15,47)*	47,99 (12,26)*	79,10 (14,98)*	50,47 (10,38)*	66,27 (13,62)*
Total sleep time	528,50 (19,08)	424,63 (34,19)*	469,45 (31,47)*	461,88 (37,08)*	331,54 (34,83)*	466,09 (36,69)*	367,28 (55,03)*	458,49 (34,72)*

Table B. Effect of CMS on time spent in wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and total sleep time during baseline and 12 h active phase and 12 h inactive phase during 7 days of CMS (*n*=13). All values are in minutes. Results are given as mean and standard deviation (±SD). Significant differences are marked with * ($p<0,001$) compared to baseline.

Active Phase	**Baseline**	**CMS Day 1**	**CMS Day 2**	**CMS Day 3**	**CMS Day 4**	**CMS Day 5**	**CMS Day 6**	**CMS Day 7**
Wakefulness								
Number of episodes	49,77 (13,54)	51,08 (8,98)	74,77 (14,82)***	70,08 (12,96)***	47,00 (13,20)	62,69 (10,19)**	61,85 (14,08)**	52,15 (15,72)
Mean episode duration	63,66 (16,03)	45,01 (9,87)**	30,39 (6,27)***	27,76 (5,71)**	65,08 (43,04)	36,56 (9,69)***	39,03 (12,06)***	54,38 (23,71)
SWS								
Number of episodes	85,77 (14,21)	103,38 (14,35)**	136,69 (17,57)***	133,31 (19,19)***	97,92 (29,86)*	123,31 (17,70)***	117,23 (24,71)***	100,23 (28,13)*
Mean episode duration	12,66 (2,24)	14,42 (1,14)*	12,08 (2,19)	14,83 (2,76)**	14,40 (3,19)*	13,61 (1,61)	14,26 (3,43)*	14,79 (3,76)**
REM sleep								
Number of episodes	13,62 (5,36)	26,85 (6,76)***	30,00 (7,60)***	29,15 (4,49)***	25,69 (8,48)***	31,15 (5,87)***	28,46 (7,15)***	21,92 (8,91)***
Mean episode duration	9,29 (1,20)	10,06 (1,22)	10,03 (1,60)	11,78 (1,80)***	11,38 (0,87)***	10,77 (1,49)**	11,06 (1,66)***	10,69 (1,41)**
Inactive Phase								
Wakefulness								
Number of episodes	60,92 (10,00)	55,46 (13,95)	66,00 (16,08)	74,31 (16,32)**	54,23 (11,57)	71,85 (19,69)*	65,08 (11,72)	71,62 (19,80)*
Mean episode duration	15,06 (2,15)	30,79 (6,48)***	21,80 (6,63)**	19,10 (3,94)	42,26 (7,07)***	19,36 (3,74)*	30,94 (8,40)***	21,43 (5,39)**
SWS								
Number of episodes	152,00 (16,90)	111,38 (13,29)***	134,62 (24,87)*	150,31 (18,43)	103,31 (15,05)***	146,00 (23,58)	125,00 (26,08)***	136,69 (27,57)*
Mean episode duration	16,93 (2,33)	18,70 (2,93)	17,62 (3,77)	15,11 (2,36)*	16,64 (2,98)	16,39 (4,81)	15,30 (2,63)	17,91 (5,14)
REM sleep								
Number of episodes	45,77 (6,83)	30,46 (5,35)***	34,38 (6,79)***	37,23 (8,64)***	21,77 (6,42)***	34,08 (6,81)***	24,46 (5,55)***	33,15 (7,84)***
Mean episode duration	12,57 (1,74)	14,50 (2,10)**	14,67 (2,69)***	13,20 (1,86)	13,10 (1,98)	13,62 (1,45)	12,39 (2,66)	11,83 (1,03)

Table C. Effect of chronic mild stress (CMS) on number of episodes and mean episode duration of wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and total sleep time during baseline and 12 h active phase and 12 h inactive phase during 7 days of CMS (*n*=13). All values are in minutes. Results are given as mean and standard deviation (±SD). Significant differences are marked with * ($p<0,05$), ** ($p<0,01$) and *** ($p<0,001$), compared to baseline.

	Interval 1	Interval 2	Interval 3	Interval 4
Baseline	**19:00-22:00**	**22:00-01:00**	**01:00-04:00**	**04:00-07:00**
Wakefulness	132,69 (17,31)	138,49 (16,75)	132,87 (24,00)	102,03 (22,07)
SWS	40,77 (13,98)	38,38 (15,06)	41,36 (20,21)	67,71 (19,19)
REM sleep	5,62 (4,80)	2,36 (2,50)	5,17 (4,75)	8,37 (4,64)
Total sleep time	46,38 (17,45)	40,74 (17,13)	46,53 (24,11)	76,08 (22,43)
Water Deprivation	**19:00-22:00**	**22:00-01:00**	**01:00-04:00**	**04:00-07:00**
Wakefulness	94,96 (19,28)***	94,31 (19,62)***	107,41 (12,09)***	85,73 (23,89)*
SWS	66,87 (13,73)	70,41 (14,86)	61,79 (8,84)	81,24 (18,78)
REM sleep	17,49 (8,39)***	14,35 (7,89)***	9,29 (5,32)	11,46 (6,06)
Total sleep time	84,36 (19,95)***	84,76 (20,48)***	71,09 (12,33)**	92,71 (24,14)*

Table D. Interaction effect of water deprivation and interval on time spent in wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and total sleep time during 12 h active phase during day 2 of CMS (*n*=13). All values are in minutes. Results are given as mean and standard deviation (±SD). Significant differences are marked with * (p<0,05), ** (p<0,01) and *** (p<0,001), compared to the corresponding time interval during baseline.

	Interval 1	Interval 2	Interval 3	Interval 4
Baseline	**07:00-10:00**	**10:00-13:00**	**13:00-16:00**	**06:00-19:00**
Wakefulness	34,60 (18,92)	29,18 (7,63)	44,36 (15,26)	69,08 (14,10)
SWS	119,88 (15,03)	119,22 (9,26)	110,64 (10,09)	81,38 (11,29)
REM sleep	24,79 (6,10)	29,28 (4,61)	24,53 (6,58)	18,77 (5,26)
Total sleep time	144,68 (18,23)	148,50 (9,12)	135,17 (15,40)	100,15 (14,56)
Continuous Light	**07:00-10:00**	**10:00-13:00**	**13:00-16:00**	**16:00-19:00**
Wakefulness	64,10 (26,66)***	87,87((25,53)***	53,86 (31,02)	49,58 (14,57)*
SWS	93,64 (19,29)**	76,99 (20,43)***	105,56 (25,29)	103,33 (12,16)**
REM sleep	21,87 (8,23)	13,45 (6,48)***	20,24 (7,10)	26,79 (8,10)**
Total sleep time	115,51 (26,68)**	90,44 (26,68)***	125,81 (31,25)	130,13 (14,48)**

Table E. Interaction effect of continuous light and interval on time spent in wakefulness, slow wave sleep (SWS) and rapid eye movement (REM) sleep, and total sleep time during 12 h inactive phase during day 3 of CMS (*n*=13). All values are in minutes. Results are given as mean and standard deviation (±SD). Significant results are marked with * (p<0,05), ** (p<0,01) and *** (p<0,001), compared to the corresponding time interval during baseline.