

# **The early life condition**

Importance for sleep, circadian rhythmicity, behaviour and  
response to later life challenges

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## Scientific environment

Working with the thesis, I have been employed as a PhD research fellow at the Department of Biological and Medical Psychology (IBMP), University of Bergen, Norway (2010-2013). The PhD grant was provided by the Norwegian Research Council Board of Mental Health as part of a translational project “Early Life Stress: A Translational Study of Affective and Behavioural Outcomes and Genetic Modulation”. I have also been employed as a trainee in sleep research at the Norwegian Competence Center for Sleep Disorders (SOVno), Haukeland University Hospital, Bergen, Norway (2008-2009) and received a writing grant from the same institution (September 2013).

The experiments in this thesis have been carried out at IBMP, where I was a member of the Research Group on Experimental and Clinical Stress and Sleep (RECSS) and associated with The International Graduate School in Integrated Neuroscience.

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## Abstract

Early life environment has a vast impact on development and adult functioning. Optimal brain development depends not only on genetic programming but also on specific external stimuli, where mother-infant relationships play an important role. In rats, through maternal presence and active care, important stimuli are provided which influence the development of behaviour and basic physiological functions in the offspring such as sleep, circadian rhythms and stress-regulating mechanisms.

Events in early life can define the development of the offspring, and depending on the events' timing and duration, may induce long-term positive or negative consequences. If the events are adverse they may induce enhanced vulnerability to stress exposure later in life. Clinical studies have revealed a close link between adverse early life events and development of affective disorders in adulthood. Underlying mechanisms are commonly studied using experimental models of early life adverse events based on daily separations of infant rats from their mothers for periods longer than considered natural, in comparison to other more natural early life conditions.

The main aim of this study was to investigate three early life conditions in rats, long maternal separations (LMS), brief maternal separations (BMS) or non-handling (NH), and their consequences in adulthood on: brain activity, sleep, circadian rhythms, levels of corticosterone, affective-like behaviour and cognitive performance. Effects of early life conditions in combination with exposure to chronic mild stress (CMS) in adulthood were also investigated. Such a combination, which yields a potentially high etiological and construct validity, has not received great attention in the preclinical literature.

Paper I investigated the consequences on brain activity measured by electroencephalogram (EEG) power and sleep in adult LMS and BMS offspring. As an effect of early life condition, adult LMS offspring showed lower EEG power recorded from the frontal cortical structures during both sleep and wakefulness,

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compared to BMS offspring. The quality of slow wave sleep (SWS) differed as a consequence of maternal separation. LMS offspring showed more deep SWS but lower power of delta waves and a slower reduction of the sleep pressure compared to BMS offspring. Exposure to CMS led to similar reductions in EEG power during sleep and wakefulness and affected reduction of sleep pressure in both groups. Compared to BMS offspring, the lower EEG power was still present in LMS offspring who also showed longer total sleep time and an indication of higher pressure for rapid eye movement (REM) sleep.

In Paper II, the consequences on circadian rhythmicity of body temperature, locomotor activity and heart rate were investigated in adult LMS and BMS offspring. As an effect of early life condition, LMS offspring showed a delayed circadian peak of body temperature compared to BMS offspring. Investigation of all other parameters showed that circadian rhythms of body temperature, locomotor activity and heart rate were similar between adult LMS and BMS offspring. The stronger impact of CMS exposure in LMS offspring was evident in stronger and longer lasting reduction of body temperature compared to BMS offspring. The degree of mothers' active care was reflected in the degree of hypothermia in LMS offspring. More active nursing following maternal separation moderated the severity of hypothermia.

In Paper III, consequences of three early life conditions, LMS, BMS and NH were investigated measuring levels of corticosterone, affective-like behaviour and cognitive performance. LMS offspring displayed higher basal level of corticosterone than BMS offspring and both NH and LMS offspring showed poor cognitive performance measured by lower object exploration compared to BMS offspring. NH also showed lower pre-pulse inhibition than LMS and BMS offspring. These results reflect adverse consequences of both LMS and the condition with constant presence of the mother. There were no differences in affective-like behaviour between the three early life conditions. Exposure to CMS induced an anhedonic-like state in all offspring. An initially high level of corticosterone was not further elevated by CMS in LMS offspring, whereas they explored objects less compared to BMS offspring. Upon CMS exposure, both BMS and NH offspring increased their object exploration.

A positive effect of CMS in NH offspring was also indicated by increased habituation and pre-pulse inhibition in acoustic startle test.

The present study describes consequences of different early life conditions (LMS, BMS or NH) on adulthood functioning and different consequences of exposure to chronic stressors in adulthood. Overall, the results indicate that exposure to LMS during early life may have adverse consequences for brain functioning as reflected in measures of brain activity and cognitive performance. Results in BMS offspring confirm that brief separations early in life may provide a “toughening up” effect in adulthood. Exposure to CMS affected brain activity in both LMS and BMS offspring. More severe impact was observed on cognitive performance and thermoregulatory response in the LMS offspring; importantly, active maternal care reduced the negative consequence of CMS. Brain activity was not assessed in the NH offspring, while the results on cognitive performance suggest adverse consequences of early life condition with the constant presence of the mother. However, remarkably, the results indicate that adult exposure to chronic mild stressors mimicking daily hassles in humans may produce a positive effect in NH offspring. Overall, the present findings reveal that different experiences and hence different developmental conditions during early life may have consequences for adulthood brain functioning.



## List of publications

- Mrdalj J., Pallesen S., Milde A.M., Jellestad F.K., Murison R., Ursin R., Bjorvatn B. and Grønli J. (2013). Early and later life stress alter brain activity and sleep in rats. *PLoS ONE* 8 (7): e69923
- Mrdalj J., Mattson, Å.L., Murison R., Jellestad F.K., Milde A.M., Pallesen S., Ursin R., Bjorvatn B. and Grønli J. (2014). Hypothermia after chronic mild stress exposure in rats with a history of postnatal maternal separations. *Chronobiology International* 31(2): 252–264
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## Abbreviations

ACTH	adrenocorticotropic hormone
AFR	animal facility rearing
ANOVA	analysis of variance
BMS	brief maternal separation
CMS	chronic mild stress
CRF	corticotropin releasing factor
ECG	electrocardiogram
EEG	electroencephalogram
EMG	electromyogram
FF	frontal-frontal
FFT	fast fourier transform
FP	frontal-parietal
HPA	hypothalamus-pituitary-adrenal
IVC	individually ventilated cages
LMS	long maternal separation
NH	non-handling
NREM	non-rapid eye movement
PND	postnatal day
REM	rapid eye movement
SCN	suprachiasmatic nucleus
SWS	slow wave sleep

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# 1. Introduction

## 1.1 Brain development

Development of an individual occurs through an interplay between genetically programmed events and external stimuli. Brain development starts during early prenatal life and continues during postnatal life, childhood and adolescence through both programmed and experience-dependent events. In general, specific neuronal connections are created and modified, some are strengthened and stabilized while others are discarded. The early postnatal period is characterized by growth of axons, dendrites and interneuronal connections, synapses. Neuronal growth and synaptogenesis are genetically programmed events. Initially, there is an overproduction of synapses which are selectively deleted during the later stages of postnatal life - synaptic pruning (Goldman-Rakic, 1987; Webb et al., 2001). Which synapses survive depends strongly on the external environment providing specific sensory input to the brain (Glaser, 2000). Neuronal connections may be discarded if not used or properly stimulated. Sensory experiences which arise from parent handling and communicating with the infant may represent important guidance for which synapses will be strengthened or discarded (Glaser, 2000).

### 1.1.1 The importance of maternal care

During early life most mammalian species are strongly dependent on maternal care for nourishment, warmth and protection. While the amount of maternal care may differ, its significance is shared among species (Kuhn & Schanberg, 1998). Through maternal presence and active care, important stimuli are provided which influence the development of behaviour and basic physiological functions in the offspring.



Figure 1: Rat mother in arched back posture while delivering milk, licking and grooming the offspring (approx. postnatal day 13).

In rats, the mother spends most of her time feeding and nursing the offspring during the first postnatal day (PND), with short periods away (e.g. 20-30 min). These periods gradually increase as the offspring become more mature (Grota & Ader, 1969). Maternal behaviour is based on the interaction between the mother and the offspring (Figure 1). Through ultrasonic vocalisation, the pups signal their presence and trigger retrieval behaviour by the mother, while the arched-back posture of the mother together with olfactory cues from her ventrum stimulate pups' attachment to the nipples and suckling. Milk is delivered in regular bouts, the mother is licking and grooming the offspring and helps maintain their body temperature (Lee & Williams, 1977). These nutritional, tactile and thermal stimuli have been termed "hidden regulators of infant physiology and behaviour" (Hofer, 1994). Their importance becomes clear in the absence of the mother for a prolonged time (e.g. 24 h at PND 14) upon which the rat offspring display several physiological and behavioural responses such as reduced heart rate, thermogenesis and growth hormone release, as well as behavioural hyperactivity and fragmented sleep (Hofer, 1994). Infant non-human primates display similar changes during a 10-day period of maternal absence (Reite et al., 1978).



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Through a line of careful experiments in rats, it was shown that each physiological response in the offspring can be closely linked to the lack of one or several specific maternal stimuli (Hofer, 1994). Accordingly, tactile stimuli, a part of active maternal care, were attributed a strong regulatory role of growth hormone release in the offspring, important for survival and differentiation of neurons and synaptogenesis. Indeed, offspring of mothers that display high amounts of active maternal care show signs of increased synaptogenesis (Liu et al., 2000b). Mimicking maternal stimuli through artificial feeding and stroking can reverse the drop in growth hormone release upon maternal separation (Kuhn & Schanberg, 1998) or prevent neuronal alterations in rat offspring reared in maternal isolation (Chatterjee et al., 2007). Furthermore, in human preterm infants, physical contact helps stabilize their sleep pattern and even reduces risk for disease (Browne, 2004).

### **1.1.2 Development of brain activity and sleep**

#### *Brain activity*

Communication between neurons, the inhibitory and excitatory membrane potentials, represent the basis of the brain electrical activity, resulting in different rhythms of slow and fast frequencies. Despite the large differences in brain size between species, brain activity is remarkably similar (Buzsaki et al., 2013). In general, slow rhythms reflect synchronous neuronal firing, whereas fast frequency rhythms reflect non-synchronous firing of many individual neurons. Brain electrical activity can be recorded via electrodes placed on the scalp providing electroencephalogram (EEG). The EEG signal can be described as a sum of electrical potentials arising in the cerebral cortex regulated via subcortical areas. EEG power analysis provides a description of the different frequencies across a given time period reflecting both the amplitude and the number of the waves.

EEG activity in new-born and young individuals is undifferentiated and only reaches adult characteristics gradually. As the brain develops through the early and later postnatal period, its reorganization and maturation are reflected in brain activity. Human studies have shown that EEG power of several frequencies first increases and

then declines with age. An increase in EEG power during the early phase of development reflects neuronal growth and increasing synaptic density. Reduced cortex thickness as a result of synaptic pruning is reflected in reduced EEG power (Somsen et al., 1997; Martinovic et al., 1998).

### *Sleep*

During sleep most of the sensory input to the cerebral cortex is actively inhibited through a complex thalamocortical interplay and generally, a slow oscillating highly synchronized EEG activity appears (Steriade et al., 1993). Sleep can be defined as a “reversible behavioural state of perceptual disengagement from and unresponsiveness to the environment“ (Carskadon & Dement, 2011). Not only is brain activity changed during the course of sleep, but so are many physiological functions such as thermoregulation, heart rate and muscle tone. One of the readily used methods for sleep recording is polysomnography, which includes recording of EEG, muscle activity (monitored by electromyogram, EMG) and eye movements (monitored by electrooculogram, EOG).

The amount of sleep and sleep characteristics change throughout the life span. While sleep length in adult humans usually is about 6-9 h, infants may spend up to 16-17 h asleep per day. Generally, the higher amount of sleep in new-born and young individuals favours brain development. As in humans, the EEG in new-born rats is undifferentiated and EEG power gradually increases during early postnatal development. The characteristic adult features for each sleep stage, such as spindles and delta waves during slow wave sleep (SWS), and homeostatic sleep regulation, are established around 3-4 weeks of age in rats (Frank & Heller, 1997; Frank et al., 1998). Accordingly, sleep staging during early postnatal life is based mostly on behavioural criteria, defining active sleep and quiet sleep. During active sleep, muscular twitches are highly abundant, often occurring in bursts. Active sleep is believed to be a precursor of rapid eye movement (REM) sleep and quiet sleep a precursor of non-rapid eye movement (NREM) sleep, although some evidence exists that both REM sleep and NREM sleep develop from active sleep (Frank & Heller, 1997).

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In human and rat infants, the proportion of REM sleep is high and declines with age (Roffwarg et al., 1966; Frank & Heller, 1997). Hence, REM sleep is believed to be important for the developing brain. A selection of neurons in the hippocampus and several cortical areas show activation directly related to muscular twitching in neonate rats (Khazipov et al., 2004; Mohns & Blumberg, 2008; Tiriach et al., 2012). These studies favour the hypothesis of high amount of REM sleep during early postnatal life being important for differentiation and maturation of developing neurons. In line with this, the proportion of REM sleep in humans declines as a result of brain maturation (Hobson, 1995). Another significant developmental change in sleep is the reduction of deep NREM sleep during adolescence (Jenni & Carskadon, 2004). Moreover, the maturation of the cerebral cortex is also reflected in sleep stage specific EEG frequencies such as reduced slow wave activity (0.5-2 Hz) which is positively correlated with the thinning of cerebral cortex caused by synaptic pruning (Buchmann et al., 2011).

### **Sleep in adult individuals**

Sleep in adult individuals is generally divided into NREM sleep and REM sleep. In humans, NREM sleep is further divided into 3 stages (N1-N3) (American Academy of Sleep Medicine, 2007). From wakefulness to sleep, high-frequency low amplitude EEG is replaced with lower frequency activity mostly in theta range (4-9 Hz), muscle tone is lowered and slow eye movements are observed (N1). N2 stage is characterized with the presence of sleep spindles (11-16 Hz) and k-complexes. Stage N3, also termed SWS, is characterized by slow wave activity (0.5-2 Hz) of high amplitude (75  $\mu$ V or higher). N3 is also termed deep sleep, as the intensity of stimuli for awakening from this sleep stage is higher than during any other sleep stage. REM sleep is characterized by low amplitude theta frequency EEG, lowest or abolished muscle tone (muscle atonia), and phasic motor activity, i.e. rapid eye movements and muscle twitches (Carskadon & Dement, 2011).

In adult rats, NREM sleep is usually termed SWS and divided into two stages (SWS1-2) (Neckelmann & Ursin, 1993). SWS1 is equivalent to N1 and N2 stages in humans, characterized by the presence of sleep spindles and less than 50% of the

EEG activity in the delta frequency range (0.5-4 Hz). SWS2 is equivalent to human deep sleep, characterized by 50% or more of the delta activity. During REM sleep there is a low amplitude theta activity and muscle atonia. NREM sleep and REM sleep alternate in cyclic fashion throughout the sleep period.

In adult humans, one NREM-REM sleep cycle lasts about 90 minutes. Hence, 4-5 sleep cycles are typically present in one sleep period (Carskadon & Dement, 2011). Shifts in sleep stages are more frequent in rats and the length of one NREM-REM sleep cycle is about 12 minutes (McCarley, 2007).

### **Sleep regulation**

Sleep is biologically regulated through the interplay between a homeostatic and a circadian factor (Borbely, 1982). The homeostatic factor represents the sleep need or sleep pressure which is built up during wakefulness. The sleep pressure is reflected in the amount of slow wave activity during NREM sleep, which is high during the early sleep period and decreases progressively. The longer the prior awake period, the higher is the EEG power of slow waves. Wakefulness is associated with increased neuronal activation and synaptic strength and it has been proposed that slow waves have a restorative function by downscaling the synaptic strength (Tononi & Cirelli, 2006). The circadian factor regulates the timing of sleep and its length. Sleep usually occurs during the declining stage of the circadian oscillation, while it is difficult to fall asleep during the increasing stage of the circadian oscillation (increasing activation). Hence, sleep depth is a function of prior wakefulness while sleep length is dependent on when sleep is initiated. In addition, sleep is also influenced by behaviour. Wakefulness can be prolonged by maintaining physical activity, intake of stimulants such as caffeine, or influenced by environmental factors, e.g. increased demands to forage for food, active avoidance of a danger such as predation.

### **1.1.3 Development of circadian rhythms**

The circadian system adapts most of our physiological functions to the cyclic variation in daylight during the 24 h. Rhythmic activity is prominent in a wide range of functions, e.g. core body temperature, activity, heart rate, hormone levels, sleep-

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wake cycles and even cognition, attention and mood (Monk et al., 1992). Humans are active during the light phase of the 24 h and some physiological functions such as body temperature and heart rate are higher during this period. In contrast to humans, rats display activity, and higher body temperature and heart rate during the dark phase (see Figure 5 in the results section). Light is the main time signal (*zeitgeber*) in many species. Endogenous rhythmic activity in the suprachiasmatic nucleus (SCN), our biological clock located in the hypothalamus, has a period longer than 24 h (Czeisler et al., 1999), reflecting the name circadian (from Latin “*circa*” – around, “*diem*” – day). The SCN receives direct light input through retinohypothalamic tract (Moore, 1973). Thus, its activity is adjusted daily to the 24 h light/dark cycle and it controls rhythmic activity in all physiological functions in the body. Through SCN connections to the pineal gland, the release of the hormone melatonin is regulated by light (Moore & Klein, 1974). Melatonin is secreted during darkness and acts as a modulator of the circadian rhythmicity in physiological processes such as thermoregulation, as well as the SCN activity itself (Stehle et al., 1989).

Endogenous rhythmic activity in the biological clock develops in the prenatal life. As it matures, the sensitivity to external signals is changed through a gradual and programmed process (Sumova et al., 2012). The entrainment of circadian rhythmicity during the prenatal life is mediated by maternal melatonin (Davis, 1997). In humans, fetal heart rate is synchronised with maternal rest-activity and body temperature rhythm, whereas upon a gradual increase in amplitude (circadian peak) the circadian rhythmicity of body temperature is evident by 6-12 weeks of postnatal age (Mirmiran et al., 2003). The mother-infant relationship seems to be important for the development of circadian rhythmicity. Compared to mother-reared animals, artificially reared infant non-human primates show different circadian profiles in body temperature, such as in lower body temperature during the inactive phase and delayed circadian peak (acrophase) (Lubach et al., 1992). In the early postnatal days, the rat SCN is immature and during this period non-photic signals act as important *zeitgebers*. Several aspects of maternal behaviour show circadian rhythmicity. The mother spends a longer time with the litter during the inactive (light) phase and displays more active nursing such as arched back posture and licking during the

active (dark) phase (Grota & Ader, 1969; Lee & Williams, 1977). A close relationship between maternal stimuli and entrainment of circadian rhythmicity in rat offspring has been demonstrated (Takahashi & Deguchi, 1983; Ohta et al., 2002). Synaptogenesis in the offsprings' retinohypothalamic tract is observed by PND 6-10, the time window when light becomes the main zeitgeber (Guldner, 1978; Duncan et al., 1986). Although opening of the eyes takes place around PND 15, the light can be received through the eyelids earlier. Also, during infancy thermoregulatory mechanisms are only efficient in a narrow ambient temperature range and within a short time period (up to 30 min) (Conklin & Heggeness, 1971; Suchecki et al., 1993a), resulting in dependence on maternal regulation to maintain normal body temperature. The age when adult patterns of circadian rhythmicity in body temperature and locomotor activity, and mature characteristics of the thermoregulatory mechanisms emerge, is around 3-4 weeks (Conklin & Heggeness, 1971; Kittrell & Satinoff, 1986).

#### **1.1.4 Development of the stress regulating mechanisms**

The activity of the hypothalamus-pituitary-adrenal (HPA) axis preserves the body's homeostasis and ensures survival if normal physiological functions are threatened and disturbed by a stressor. In brief, corticotropin releasing factor (CRF) secreted from the paraventricular nucleus of the hypothalamus stimulates release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland. ACTH acts on the adrenal cortex to release glucocorticoids (corticosterone in rodents, cortisol in primates) which exert widespread effects in the body to restore homeostasis. The activity of HPA axis is dampened through negative feedback mechanisms, mediated mainly via corticoid receptors in the hippocampus (Lopez et al., 1999).

Stress regulating mechanisms also undergo significant maturational changes during brain development. During early PNDs in rodents, the feedback mechanism is immature. The period between PND 2-14 is termed the stress hyporesponsive period as the levels of corticosterone remain relatively low even if the pups are presented to stimuli which normally elicit an increase of corticosterone in adult rats (e.g. handling,

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exposure to novelty) (Levine, 1994). Sustaining glucocorticoids at low levels seems to be critical for normal growth, differentiation and maturation of neurons (Bohn, 1980). It has been demonstrated that maintenance of the stress hyporesponsive period depends strongly on maternal care, where tactile and nutritional stimuli inhibit over-secretion of ACTH and corticosterone (Suchecki et al., 1993b; Levine, 1994). Moreover, high levels of maternal licking and grooming can strengthen the negative feedback mechanism in the offspring (Liu et al., 1997). Whether these mechanisms exist in the developing HPA axis of humans is not clear, yet there are indications of stress hyporesponsivity during the first two postnatal years, which is related to the quality of caregiving provided to the infant (Gunnar & Cheatham, 2003).

## 1.2 Early life events

The evidence that optimal brain development depends not only on genetic programming but also on specific external stimuli highlights the importance of the environment during early life for normal brain development. As a consequence of adverse early life events, changes in brain development might be long-lasting and affect the individual's functioning in adulthood. Many children live in homes or institutions under deprived conditions that do not provide sufficient motor, cognitive or social stimulation (Guler et al., 2012). A World Health Organization survey across 18 countries identified a high prevalence (up to 38%) of adverse childhood events such as family economic adversity, loss of a parent, parental divorce, neglect, physical or emotional abuse (Kessler et al., 2010). Approximately 1.2 million children in USA fall victim to maltreatment according to another recent report (Sedlack et al., 2010). Experiencing such events during early life can have negative consequences for adult physical and mental health. Hence, understanding the underlying mechanisms which may affect adulthood functioning is important. Clinical studies are usually based on retrospective reports and thus lack the power in delineating specific cause-effect relationships. Longitudinal studies may be limited by complexity of different factors.

### **1.2.1 Animal models of early life adverse events**

Studies on animals have become invaluable by enabling controlled experimental manipulations that would be unethical to perform in humans, and investigating their consequences. One purpose of using animal models is to ensure our basic understanding of normal physiological functions. Another purpose is to study the underlying mechanisms and pathophysiology of a specific disorder. Animal models also serve as a tool to study mechanisms of action of specific drugs. The knowledge gained and translated from animal models may advance human welfare.

Animal models of early life adverse events are numerous. One of the more commonly used experimental models of early life adverse events is daily separations of infant rats or non-human primates from their mothers for periods longer than considered natural (e.g. 3-6 h, from now on termed long maternal separations, LMS). Such manipulations result in repeated discontinuation of maternal care. LMS is usually compared to early life conditions either involving brief maternal separations (BMS; e.g. 10-15 min) and/or rearing in the constant presence of the mother (no separation and non-handling, NH; or no separation but standard animal facility rearing, AFR) (Plotsky & Meaney, 1993; Caldji et al., 2000; Lehmann & Feldon, 2000).

Brief separation closely mimics a natural caregiving environment where the mother displays short periods away from the offspring to forage. Mothers left undisturbed with their offspring, such as in the NH condition, may be intuitively thought to represent a safe and stable rearing environment. However, the mothers' active maternal care may not come naturally since her environment lacks the natural triggers to evoke maternal response. Hence, such an early life environment may also be inadequate for the normal maturation of neurobiological systems in the offspring. The mother is constantly present and the stress regulating mechanisms are not challenged adequately during development. In line with this it has been hypothesized that the NH condition may result in offspring with a similar phenotype to that of LMS (Caldji et al., 2000; de Jongh et al., 2005; Madruga et al., 2006). Yet, studies based on manipulations of mother-infant environment frequently describe NH as a control



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group even though the natural caregiving environment in the NH condition may not be appropriate. Clearly, BMS, LMS and NH should be considered as three different early life conditions and compared in terms of their effects on adult functioning.

Other models of early life stress may be based on naturally occurring low levels of active maternal care in rats (licking and grooming and arched-back nursing) (Liu et al., 1997), separating the offspring for a single prolonged period (24 h) (Stanton et al., 1988) or exposing the offspring to daily separations from both mother and littermates (1-6 h), resulting in early deprivation (Pryce & Feldon, 2003). In non-human primates, maternal deprivation is achieved through early separation from the mother and subsequent artificial rearing (Pryce et al., 2011). Yet another approach to adverse early life events is to expose the mother to an environmental stressor, such as providing limited nesting material (rats) (Brunson et al., 2005), or increasing the demand for food seeking (non-human primates) (Levine & Mody, 2003), manipulations which result in inconsistent maternal behaviour.

## **1.2.2 Consequences of adverse early life events**

### *Brain structures and function*

Inadequate or inappropriate stimulation during early life can influence normal brain development negatively. Children raised in deprived conditions such as orphanages may show signs of developmental delay, including reduced grey and white matter volume (Sheridan et al., 2012). Studies in adults with a history of adverse childhood events have found decreased volume of limbic and cortical brain areas and interhemispheric connections (*corpus callosum*) (Teicher et al., 2006; Pechtel & Pizzagalli, 2011). Reduced white matter volume following early life adverse events in non-human primates has been related to elevated levels of cortisol, hence underlining the importance of maintaining low levels of cortisol during development (Howell et al., 2013). Similar structural changes observed in the frontal cortex and hippocampus after LMS (3 - 6 h) or 24 h separation in rats have been attributed to impaired neurogenesis, decreased number of synapses, altered monoaminergic innervation and glial structure, or decreased number of granule cells and markers of neural plasticity

(Andersen & Teicher, 2004; Mirescu et al., 2004; Leventopoulos et al., 2007; Aisa et al., 2009a; Hulshof et al., 2011; Chocyk et al., 2013; Ohta et al., 2013). Together these studies provide evidence that adverse early life events can affect the developing brain, influencing its reorganization and maturation.

Effects on brain structure associated with early life stress may be reflected in brain activity and function (Pechtel & Pizzagalli, 2011). Alterations in EEG activity during wakefulness and poor cognitive functioning have been found in children raised in institutions compared to those who were adopted early or family raised, as well as in adults that had been exposed to adverse early life events such as physical abuse (McFarlane et al., 2005; Hedges & Woon, 2010; Tarullo et al., 2011; Guler et al., 2012). In rats, cognitive performance may be investigated by measuring object exploration, recognition of new objects or searching for a hidden platform in a water maze. Studies involving these assessments have indicated a reduced cognitive performance, such as in reduced object exploration time and increased latency to find the hidden platform in the water maze, in rats after LMS (3 h) or low maternal care. These observations were associated with altered neuronal mechanisms of memory formation (Bredy et al., 2003; Champagne et al., 2008; Aisa et al., 2009a; Chocyk et al., 2013). Some studies have shown similar cognitive performance in adult LMS compared to NH or AFR offspring (Mourlon et al., 2010; Vivinetto et al., 2013). Pre-pulse inhibition represents a behavioural neurobiological measure, a measure of sensorimotor gating. Whether different early life conditions change the ability to utilize pre-pulse inhibition requires further investigation, especially given the divergent results described: no changes or increase after LMS (6 h) or early deprivation (Lehmann et al., 2000; Pryce et al., 2001; Li et al., 2013).

Thus, changes in brain activity and findings of altered cognitive performance and behaviour seem to reflect negative effects on brain development. Nevertheless, investigation of brain activity such as measured by EEG has not received great attention in animal models of adverse early life events. Moreover, the link between alterations in cognitive performance and changes in EEG has not yet been addressed.

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### *Sleep and circadian rhythms*

Sleep disturbances such as insomnia, increased nightly awakenings, nightmare-related distress and restless sleep are frequent subjective complaints amongst adult humans with a history of childhood adversities (Csoka et al., 2011; Steine et al., 2012a). However, only one previous study performed sleep EEG recording and found less deep NREM sleep in subjects reporting high versus low levels of adverse early life events (Bader et al., 2007). Whether sleep homeostasis is impaired after adverse childhood experiences has not been investigated in clinical or preclinical studies. Furthermore, the effects of early life adverse events on sleep and EEG have received little attention in the preclinical literature. One study described an increased number of awakenings in young non-human primates exposed to LMS (30 min – 2 h) (Pryce et al., 2011). In rats, prenatal stress or postnatal LMS (3 h or 6 h) affected quantity and quality of sleep in adulthood (Rao et al., 1999; Tiba et al., 2004; Feng et al., 2007). However, the results are divergent, e.g. both increase and decrease in REM sleep have been described (Feng et al., 2007; Feng et al., 2012), which urge for further investigations.

Abnormalities in circadian regulation may result in a system that is less adaptable to environmental variations and thus contribute to the development of psychiatric disorders (Kronfeld-Schor & Einat, 2012). Yet, most studies on early life adverse events, both clinical and preclinical, do not measure circadian rhythmicity. An increase in activity levels during the inactive phase of the 24 h cycle has been associated with childhood abuse in both children and adults (Glod et al., 1997; Bader et al., 2007). No clinical reports exist on circadian rhythms of body temperature and heart rate after early life adverse events. Rats with a history of maternal deprivation (12 h), early weaning or juvenile stress show alterations in locomotor activity and body temperature upon adulthood stress exposure (Ackerman et al., 1978; Yoshihara et al., 2005; Yee et al., 2011). The circadian rhythmicity of body temperature has not been investigated after LMS, while studies measuring locomotor activity and heart rate showed no circadian effect (Feng et al., 2012; Trombini et al., 2012).

Studies on sleep and circadian rhythmicity after early life stress require further attention, particularly given that disturbed sleep and circadian dysregulation are common symptoms in many psychiatric disorders and may also be involved in their aetiology (Lustberg & Reynolds Iii, 2000; Riemann & Voderholzer, 2003; Germain & Kupfer, 2008). Moreover, sleep EEG abnormalities associated with depression have also been found in humans with a high risk for depression (Fulton et al., 2000; Friess et al., 2008). This highlights the need for investigating sleep EEG after early life adverse events.

### *HPA axis and increased stress responsivity later in life*

One of the most frequently observed consequences associated with early life adverse events is an increased responsivity to stressful events later in life. Adult rats with a history of LMS (3 h) show several indicators of HPA axis hyperactivity upon exposure to an acute stressor in adulthood (e.g. restraint (Plotsky & Meaney, 1993; Liu et al., 2000a)). Increased levels of CRF, ACTH and corticosterone were found compared to AFR, NH or BMS (15 min) rats (Plotsky & Meaney, 1993; Liu et al., 2000a; Huot et al., 2001; Aisa et al., 2007). In some studies, NH offspring may show similar or even higher stress reactivity compared to LMS offspring (Plotsky & Meaney, 1993; Pryce et al., 2001; Hulshof et al., 2011). Similar hyperactivity has been described in artificially reared non-human primates. As adults they showed higher levels of ACTH and cortisol and increased behavioural responses to social separation stressors, compared to mother-reared individuals (Suomi, 1991). In adult humans with a history of childhood abuse, exposure to public speaking, used as a psychosocial challenge, led to a stronger response in the HPA axis (increased ACTH) and elevated heart rate compared to non-abused individuals (Heim et al., 2001).

The increased stress responsivity associated with adverse childhood experiences may be explained by structural and functional alterations in the HPA axis. A reduced number of corticoid receptors in several brain regions indicates an altered negative feedback mechanism. Such an outcome has been identified across species: in rats after LMS (3 h) or reared in low maternal care condition, in non-human primates after LMS (30 min – 2 h) and in humans with a history of adverse childhood events

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(Liu et al., 1997; Ladd et al., 2004; McGowan et al., 2009; Arabadzisz et al., 2010). Furthermore, rats with a history of LMS (3 h) or low maternal care, and non-human primates reared by mothers with increased food seeking demands, show over-secretion of CRF under basal conditions (Plotsky & Meaney, 1993; Coplan et al., 1996; Caldji et al., 1998). Brain regions with a strong modulating effect on paraventricular nucleus in hypothalamus (i.e. noradrenergic connections from the locus coeruleus) may show hyperactivity and thus contribute to increased release of CRF (Liu et al., 2000a).

Altered negative feedback regulation and prolonged high levels of glucocorticoids may also result in hypocortisolism, a state associated with stress-related disorders in humans (Heim et al., 2000a; Fries et al., 2005). Low levels and altered circadian rhythm of cortisol have been described in both institutionalized children and adolescents with a history of low parental care (Carlson & Earls, 1997; Engert et al., 2011). HPA axis alterations and increased stress reactivity are one of the suggested mechanisms that link early life adverse events and development of psychopathology in adulthood (Heim et al., 2008).

### *Increased risk of psychopathology*

Epidemiological studies have indicated a close link between adverse childhood events and a variety of psychiatric disorders later in life (Heim & Nemeroff, 2001; Kessler et al., 2010). Symptoms of affective disorders, such as depression and anxiety, are more frequent among adults with a history of childhood abuse than in adults with no history of abuse (McCauley et al., 1997). Moreover, stressful life events are associated with the manifestation and worsening of depression (Kessler, 1997). It has been suggested that some individuals may become more vulnerable to stressful exposure later in life which in turn may increase the risk for developing a psychiatric disorder (Heim & Nemeroff, 2001). The increased vulnerability may be explained by structural and functional brain alterations, particularly stress regulating mechanisms, associated with early life adverse events. In some individuals, exposure to early adverse events may increase the risk for psychopathology due to a pre-existing genetic vulnerability (Drury et al., 2010; Heim et al., 2010).

**Affective disorders**

The high prevalence of affective disorders, 7.8% for major depression and 14.0% for anxiety disorders, represents a considerable social and economic challenge worldwide (World Health Organization, 2008; Wittchen et al., 2011). Major depression is a complex disorder characterized by loss of interest and pleasure (anhedonia). Other symptoms can be increased or decreased activity (psychomotor agitation or retardation), weight loss or weight gain and disturbed sleep (American Psychiatric Association, 2013). Often reported sleep complaints include difficulties initiating sleep, frequent awakenings, early morning awakening and non-restorative sleep, but also an increased need for sleep or hypersomnia. Sleep EEG recordings reveal prolonged sleep onset latency, increased wakefulness after sleep onset and early morning awakening, as well as an increased amount of REM sleep and reduced amount of deep NREM sleep (Peterson & Benca, 2011). Another reported NREM sleep abnormality in depression is reduced EEG power of slow delta waves, attributed to disturbed homeostatic regulation of sleep (Borbely et al., 1984). Depression is also associated with altered circadian rhythmicity, such as reduced amplitude of the body temperature or rest-activity rhythm (Souetre et al., 1989; Armitage et al., 2004).

Depression is highly comorbid with generalized anxiety disorder of which the core symptoms are prolonged feelings of anxiety and worry. Among other symptoms are restlessness, concentration difficulties, altered activity levels and increased startle responses (Judd et al., 1998; American Psychiatric Association, 2013). Sleep disturbance is characterized by difficulty initiating and maintaining sleep and non-restorative sleep. These subjective complaints are reflected in sleep EEG as prolonged sleep onset latency, increased wakefulness after sleep onset and reduced total amount of sleep. NREM sleep and REM sleep changes do not seem to characterize anxiety, yet given the high comorbidity rates, these sleep EEG abnormalities associated with depression may be present (Ramshaw et al., 2011). Hyperactivity in the HPA axis, such as increased CRF and/or cortisol secretion may be another pathophysiological marker in both depression and anxiety (Arborelius et al., 1999; Heim et al., 2008).

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### **Affective-like behaviour in animal models**

Behavioural tests are readily used in animal models to assess affective-like changes (Prut & Belzung, 2003; Anisman & Matheson, 2005). Upon exposure to a novel environment such as open field arena, rats with a history of LMS (3 h) or low maternal care may display low activity (Caldji et al., 1998; Huot et al., 2001; Francis et al., 2002). Low activity in the open field may be interpreted as affective-like behaviour. Similar behaviour has also been reported in adult NH offspring (Pryce et al., 2001; Shalev & Kafkafi, 2002; Madruga et al., 2006). Further, exposure to LMS (3 h) or early deprivation may result in reduced reward behaviour such as reduced preferences for sweet solutions (Hui et al., 2011; Zhang et al., 2013a), although findings of no difference or increase have also been reported (Mourlon et al., 2010; Uchida et al., 2010; Oines et al., 2012; Zhang et al., 2013b). Since rats usually prefer drinking sweet solutions to water, the reduced preference for sucrose is interpreted as a loss of interest in pleasure, hence resembling the human state of anhedonia. These behavioural abnormalities have been shown to normalize after chronic treatment with antidepressants and anxiolytics (Huot et al., 2001; Maciag et al., 2002). Alterations in serotonergic neurotransmission, as one of the possible underlying mechanisms in depression, have also been described after LMS (3 h or 6 h) and may contribute to increased stress responsivity (Arborelius & Eklund, 2007; Lambas-Senas et al., 2009; Ohta et al., 2013). Anxiety-like behaviour as indicated by reduced exploration of the central zone in the open field arena, in the open arms of the elevated plus-maze arena or enhanced acoustic startle response (an innate reflex reaction to a loud and sudden auditory stimulus (Koch, 1999)), has been found in LMS (3 h) compared to BMS, NH or AFR offspring (Huot et al., 2001; Francis et al., 2002; Kalinichev et al., 2002; Lippmann et al., 2007; Oines et al., 2012). Some studies report enhanced startle response in adult NH offspring (Caldji et al., 2000; Pryce et al., 2001).

Being closely associated with increased risk of affective disorders in humans, exposure to stressful events during adult life is frequently utilized in animal models of depression and/or anxiety. In rats, experience of a single social challenge such as defeat by a dominant conspecific may induce affective-like changes as indicated by reduced sucrose preference, low activity in the open field or elevated plus-maze,

reduced circadian amplitude of body temperature and fragmented sleep (Meerlo et al., 1996; Kinn et al., 2008; Kinn Rød et al., 2012). Exposure to chronic stress such as a series of unescapable electrical footshocks results in learned helplessness in some animals, i.e. no attempt to avoid subsequent escapable shocks (Seligman & Beagley, 1975). Chronic variable stress is based on exposure to different stressors over several days or weeks, involving relatively severe stressors such as 46 h of food or water deprivation, cold swim and footshock (Katz et al., 1981). On the other hand, the chronic mild stress (CMS) model is based on unpredictable, sequential presentations of different mild stressors (e.g. 24 h food or water deprivation, wet cage, social stress), aiming to resemble a more realistic experience of everyday life hassles in the adult life of humans (Willner et al., 1987). Chronic stress paradigms have been shown to induce affective-like changes such as weight loss, reduced open field activity and reduced intake or preference for sucrose (Vollmayr & Henn, 2003). Increased levels of corticosterone were found in rats showing learned helplessness whereas studies utilizing CMS reported both increases and decreases in corticosterone levels (Murison & Hansen, 2001; Vollmayr & Henn, 2003; Ushijima et al., 2006; de Andrade et al., 2013). In addition, CMS has been shown to induce sleep disturbances typical of human depression, increased amount of REM sleep and increased sleep fragmentation as well as altered circadian rhythms of body temperature and activity (Gorka et al., 1996; Grønli et al., 2004; Ushijima et al., 2006; Grønli et al., 2007).

Given that early life adverse events may induce enhanced vulnerability to stress exposure later in life, and the close link between stressful life events and affective disorders, experimental studies involving combinations of early life events and adult chronic stress seem highly warranted. Nevertheless, only a few studies with such an approach have so far been conducted. Exposure to chronic stress involving severe stressors induces alterations in HPA axis activity and affective-like behaviour in rats with a history of LMS (3 h or 4.5 h) compared to AFR or BMS rats (Ladd et al., 2005; Renard et al., 2005; Renard et al., 2007). Exposure to CMS increased anhedonic-like phenotype in NH rats compared to BMS (10 min), as well as in rats reared in low versus high maternal care (Henningsen et al., 2012; Boufleur et al.,



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2013). Exposure to CMS has not yet been investigated in rats with a history of LMS nor compared between the three early life conditions (LMS, BMS and NH).

### **1.2.3 Moderating factors of early life events**

The effects of early life events on individual's health and functioning in adulthood may depend on several factors such as age when the experience occurred, frequency and severity of the stressor, as well as environmental variations (Lupien et al., 2009). Several preclinical studies suggest that exposure to brief handling or brief separations from the mother (15 min) during early life may be beneficial upon exposure to a physiological or psychological challenge in adulthood. Rats that are exposed to brief separations show less anxiety-like behaviour and decreased responsivity in the HPA axis in adulthood and better cognitive performance compared to NH rats (Levine, 1957; Meaney et al., 1988; Caldji et al., 2000). This is attributed to a positive effect of short-term activation of the HPA axis during the developmental period (Meaney et al., 1985; Plotsky & Meaney, 1993). Brief separations from the mother may increase the number of corticoid receptors and thus strengthen the negative feedback mechanism of the HPA axis, a phenomenon attributed to increased maternal licking and grooming triggered by brief separations (Meaney et al., 1988). If mothers of non-human primates only occasionally are challenged with increasing food seeking demands, resulting in brief separations, their offspring show lower levels of anxiety-like behaviour and HPA axis activity than offspring without BMS experience (Levine & Mody, 2003). Hence, early life in the constant presence of the mother (NH or AFR) may "deprive" the offspring of stimuli important for normal development of the stress response and robustness. Indeed offspring reared under such conditions can display phenotypes similar to LMS offspring such as increased affective-like behaviour and decreased cognitive performance (de Jongh et al., 2005; Madruga et al., 2006; Mourlon et al., 2010). These offspring may very well benefit from stimuli that induce short-term triggering important for the stress response and robustness in adulthood, however this remains to be investigated.

Experiencing adverse events during early life increases the risk for developing a variety of disorders; however, clinical studies reveal that many individuals remain unaffected. For example, not all individuals with a history of adverse childhood events develop a psychiatric disorder (Stevenson, 1999). It seems that other experiences during or subsequent to stress exposure may have a protective effect, such as having a stable caregiver (Kaufman & Henrich, 2000). Among adults with a history of childhood abuse, one study showed that perceived parental care as well as quality of relationship to others during adolescence and adulthood were strongly associated with the absence of a psychiatric disorder in adulthood (Collishaw et al., 2007). Another study linked higher levels of perceived social support to fewer symptoms of disturbed sleep (Steine et al., 2012b).

Naturally occurring high levels of active maternal care in rats have been shown to have long-lasting positive effects on the behaviour of the offspring and their stress response in adulthood, effects that are associated with the stronger negative feedback regulation of the HPA axis (Liu et al., 1997; Francis et al., 1999). Moreover, high amounts of active maternal care are associated with improved cognitive performance in adulthood (Liu et al., 2000b). In humans, variations in HPA axis response to a psychosocial challenge in adulthood may also be related to perceived amount of maternal care during early life (Engert et al., 2010). Furthermore, studies in rats have shown that providing environmental enrichment to the offspring during adolescence may reverse the negative consequences of early life adverse events. Socially and environmentally enriched housing after weaning prevents both the increase in HPA axis responsivity and affective-like behaviour in adult rats that have been exposed to LMS (3 h) (Francis et al., 2002; Hui et al., 2011). In rats that had received low levels of active maternal care during development, subsequent environmental enrichment improved their cognitive performance (Bredy et al., 2003).

Together, animal studies support the findings in human infants where negative effects of living in deprived conditions can be reversed by providing improved caregiving and environmental stimuli through adoption and foster care (Marshall et al., 2008; Loman et al., 2010; Sheridan et al., 2012).

## 1.3 Research aims and hypotheses

Given that early life adverse events may induce enhanced vulnerability to stress exposure later in life, and the close link between stressful life events and affective disorders, experimental studies involving combinations of early life events and adult daily stressors seem highly warranted. Combining the maternal separation model with exposure to later life chronic mild stress may be one approach with potentially high etiological and construct validity within the research on early-life manipulation.

The main aims of this thesis were: 1) to investigate adult consequences of different early life conditions in rats (LMS, BMS or NH) on EEG, sleep, circadian rhythms, levels of corticosterone, affective-like behaviour and cognitive performance, and 2) to study the consequences of exposure to chronic unpredictable mild stressors (CMS) in adult rats reared under the different early life conditions.

### **Paper I**

The aims in Paper I were to investigate consequences of LMS and BMS on EEG concerning: 1) sleep-specific and wake-specific frequencies from frontal-frontal (FF) and frontal-parietal (FP) derivations and 2) sleep homeostasis. In addition, changes in sleep architecture were examined. Furthermore, a specific aim was to examine whether LMS predisposes offspring to be more susceptible to CMS in terms of more reduced brain activity, impaired sleep homeostasis and more disturbed sleep than BMS.

### **Paper II**

The aims in Paper II were to investigate 1) if LMS or BMS in rats affect the adult circadian rhythms of body temperature, locomotor activity and heart rate differently, 2) if exposure to a CMS protocol in adulthood alters the diurnal rhythmicity of the LMS rats more than BMS rats, 3) to what extent would different stressors applied add to changes in the circadian parameters during the second week of the CMS protocol, and 4) if levels of maternal care following maternal separations are associated with changes in the circadian rhythmicity induced by CMS, and with pre CMS levels of corticosterone.

**Paper III**

The aims in Paper III were to investigate 1) the adult consequences of all three early life conditions (LMS, BMS and NH); and 2) the interaction of early life condition and CMS exposure. The aims were addressed by comparing: corticosterone, affective-like behaviour and cognitive performance. As a consequence of different early life conditions it was hypothesized that LMS offspring would show increased levels of corticosterone, increased affective-like behaviour and reduced cognitive performance compared to BMS, and show no differences compared to NH offspring. Further, upon exposure to CMS it was hypothesized that LMS offspring would show decrease in basal corticosterone in LMS offspring compared to BMS and NH offspring; and LMS and NH offspring would display increased affective-like behaviors and reduced cognitive performance compared to BMS offspring.

## 2. Methods

For more detailed description of the procedures, see materials and methods section in Papers I, II and III.

### 2.1 Ethics

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.

The experiments were designed in line with the principles of “3 R’s”: reduction, refinement and replacement (Russel & Burch, 1959). Combining the use of resource equation (Mead et al., 2012) and previous experience with the models, the number of animals was reduced to a minimum, whilst still ensuring sufficient data for statistical analysis. Furthermore, 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 as animals are undisturbed in their home cages and their discomfort reduced to a minimum. The method also allowed recording of several additional parameters (body temperature, heart rate, activity) thus further reducing the number of animals used. Replacement stands for use of an alternative method (e.g. *in vitro* method), although in order to study the impact of early life events on physiological function and behaviour, *in vivo* experiments are the only methods available at the present time.

### 2.2 Animals and housing procedures

To avoid possible exposure to stress during pregnancy due to transportation, the breeding procedures were performed at the local animal facility. Female rats of Wistar strain (NTac:WH) were purchased from Taconic, Denmark, and used for breeding. Ten females gave birth to 120 offspring in total of which 64 male offspring

were included in this thesis. They were group housed in individually ventilated cages (IVC) type IV before and after weaning at PND 22, and individually in type III cages after surgical procedures at PND 56 (Figure 2). All animals had *ad libitum* access to food and water except when tested for sucrose intake and during food or water deprivation as part of the CMS protocol (see below).

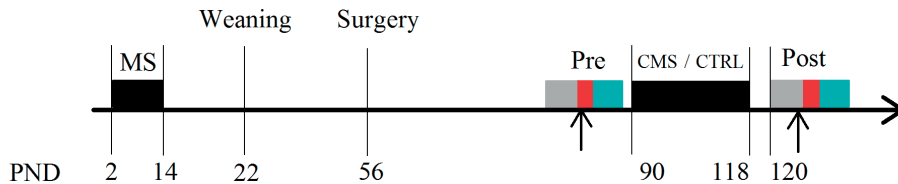


Figure 2. Experimental design. Brief or long maternal separations (MS) were performed during postnatal days (PND) 2-14. The non-handled group was undisturbed during this period. Telemetric recording (grey boxes), blood samples (red boxes), sucrose preference (arrows) and behavioural assessment (blue boxes) were performed pre and post chronic mild stress (CMS) exposure or control (CTRL) condition. See more details in Papers I, II and III.

## 2.3 Early life conditions

The day of birth was defined as PND 0. Cages were not cleaned before PND 15. Separation procedures were performed from PND 2 -14 (Figure 2); the mother was first removed from its home cage and placed in a separate cage with food and water *ad libitum*. The whole litter was then moved to a separate room and placed in a cage with chopped wood bedding and soft paper. The mother and its offspring were reunited in reverse order.

### 2.3.1 Long maternal separation

The offspring were separated daily from their mothers for one period of 180 min starting at 09:00 A.M. Ambient temperature for the pups was monitored and regulated artificially by a heating lamp (PND 2-7: 32-34 °C, PND 8-14: 28-30 °C).

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### **2.3.2 Brief maternal separation**

The offspring and the mother were separated daily for one period of 10 min starting at 09:00 A.M. For these offspring artificial heating was not provided based on an earlier report that pups can maintain their body temperature for up to 30 min in the absence of the mother (Suchecki et al., 1993a).

### **2.3.3 Maternal behaviour at reunion**

During the PND 2-7 maternal behaviour was monitored for a period of 30 min following reunion in the LMS and BMS groups. The duration of active care was recorded, i.e.: 1) mother licking and grooming at least one pup, and 2) mother nursing the pups in the arched-back posture (Champagne et al., 2003).

### **2.3.4 Non-handling**

The offspring were not exposed to the separation procedure and were left undisturbed with their mother.

## **2.4 Chronic mild stress procedure**

At PND 90 half of the LMS, BMS and NH offspring were housed in a separate room and exposed to CMS for 4 weeks (Figure 2). The remaining offspring were given normal facility rearing consisting of cage cleaning and food and water replenishment once per week (control condition).

The CMS procedure consisted of exposure to one or more stressors each day of the 4 week period. Timing and duration of the stressors are presented in Paper I and III (Table 1, both). Each week consisted of: one period of 21 h in cage without bedding, followed by 3 h exposure to 3 cm of water in the same cage; one 18 h period of food deprivation, followed by 1 h of restricted access to food (4-5 45 mg food pellets); two periods of water deprivation (16 h and 20 h) followed by 1 h exposure to an empty water bottle; two 3 h periods with cage tilted at an angle of 45 %; one 20 h period with wet bedding (bedding soaked with 300 ml of water at room temperature); one

continuous light period of 36 h; and a 2 h exposure to social stress (two rats paired per cage).

## 2.5 Surgical procedure

Surgical procedures were performed in all rats. Eight LMS-CMS and 8 BMS-CMS rats (Papers I and II) were implanted subcutaneously (s.c.) with a transmitter. All of the remaining animals underwent the same surgical protocol without implantation of the transmitter. Surgery was performed under hypnorm-dormicum anaesthesia. The rats were positioned in a stereotaxic apparatus and surgical incisions were made over the lower back, head and neck, and along the shoulder muscle. The transmitter was placed in a “saddleback” position, two electrodes were attached to the skull for EEG recording and two electrode pairs were attached to muscle for EMG and electrocardiogram (ECG) recording. The EEG leads were placed in FF and FP position with the following coordinates (Figure 3): 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). The skin was closed with sutures. To minimize the risk of infections, antibiotics were administered in drinking water one day before surgery and during the first two postoperative days. Postoperative analgesia was given twice a day for 3 days. A minimum of 14 days was allowed for recovery and to regain the preoperative weight.

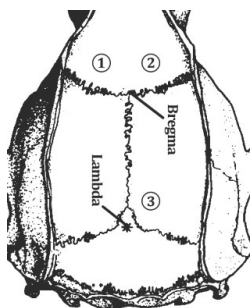


Figure 3. Placement of electrodes for electroencephalogram recording. 1: reference electrode; 1+2: frontal-frontal (FF) derivation; 1+3: frontal-parietal (FP) derivation. Illustration modified from Paxinos & Watson (1998).



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## 2.6 Telemetric signal recording and analysis

To initiate telemetric recording, the transmitter was turned on by swiping a magnet along the rat's lower back. Through a receiver placed directly under the home cage signals were collected and transferred to the acquisition software Dataquest ART (version 4.1, Data Sciences International). Sampling rates were set at 250 Hz for EEG and EMG signals, 50 Hz for body temperature data and 1000 Hz for the ECG signal. Home cage related spontaneous locomotor activity was recorded as counts/minute.

### 2.6.1 Sleep staging and EEG power analysis

Scoring of sleep stages and power spectrum analysis were performed offline with Neuroscore software (version 2.0.1, Data Sciences International) in 10 s epochs. Analyses were applied during the 12 h inactive phase (7:00 A.M. – 7:00 P.M.) pre and post CMS (Figure 2). EEG signals were low-pass filtered at 35 Hz. For qualitative scoring of SWS, based on Neckelmann & Ursin (1993), a high pass filter at 0.5 Hz for FP EEG and 3 Hz for FF EEG were used. The EMG signal was high-pass filtered at 5 Hz to remove low-frequency artefacts. Each 10 s epoch of the 12 h recording period was scored and the duration (min) of each sleep stage calculated. The following criteria were used: wakefulness: high-frequency low voltage EEG activity and high to moderate EMG activity; SWS1: spindle activity (11-16 Hz) in the FF channel, <50% of delta (0.5-4 Hz) activity in the FP channel, and EMG reduced compared to wakefulness; SWS2: spindle activity present in FF and  $\geq$ 50% of delta activity in FP channel and EMG activity equal to or lower than SWS1; REM sleep: theta (6-9 Hz) activity in the FP channel and muscle atonia in the EMG.

For each sleep stage the EEG power of the frequency bands characteristic for the stage was calculated using an offline fast fourier transform (FFT) analysis on unfiltered EEG signals in 10 s intervals (artefact-free epochs were excluded from the analysis; for more details on the procedure see Paper I). The following frequencies were analysed: SWS (SWS1 + SWS2): delta (0.5-4.4 Hz) and theta (5.5-9.4 Hz) band; REM sleep: theta, beta (19.5-34.4 Hz) and gamma (lower range, 35-60 Hz)

band; wakefulness: beta and gamma band. For investigation of the homeostatic sleep regulation an average of low range delta (0.5-2.4 Hz) power during SWS was calculated from the FP derivation for each hour after lights on at 07:00 A.M.

## **2.6.2 Circadian rhythm analysis**

Body temperature, locomotor activity and heart rate were analysed with Chronos-Fit software (Zuther et al., 2009). The 24 h analyses were performed for one recording day before CMS (pre) and two recording days after (post 1 and post 2) (Figure 2). Mean values were assessed using linear analysis: mean 24 h, 12 h active (dark) and 12 h inactive (light) phase. A partial FFT analysis of the data was performed to assess the circadian rhythm parameters: percentage of rhythmicity (indicating how well the data set fits a cosine curve), maximum and minimum values, nadir (time of minimum), amplitude (circadian peak value) and acrophase (time of circadian peak).

## **2.7 Blood sampling and corticosterone analysis**

Blood samples were collected (Figure 2) in order to determine the levels of corticosterone. The samples were collected during the rats' inactive phase (09:00 - 12:00 A.M.). Each rat was transported from the housing room to the sampling room in the home cage and anaesthetized with isoflurane vapour. The *vena saphena* was punctured and blood was collected within  $2.5 \pm 0.5$  min from the start of the transport. Samples were centrifuged and serum was extracted for analysis of corticosterone levels using enzyme immunoassay kit.

## **2.8 Behavioural tests**

### **2.8.1 Sucrose preference**

One water bottle and one bottle with sucrose solution (1%) were placed inside the rats' cages. The rats were first adapted to the sucrose solution for 48 h prior to the testing. The sucrose preference test was performed one day before and one day after the end of the CMS procedure (Figure 2). Liquid consumption was measured by

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comparing the weight of the bottles before and at the end of the active (dark) phase. Sucrose preference was calculated using the formula: sucrose consumption / total liquid consumption.

### **2.8.2 Open field**

A black, squared apparatus sized 100 x 100 cm and 40 cm high walls was used for open field test. The area more than 20 cm from the walls was defined as the central zone of the field. To prevent odour cues interfering with exploration, the apparatus was cleaned with a 5% ethanol solution between each test. The rat was placed in the central zone and allowed to explore for 9 min. Its activity was monitored by a camera placed above the apparatus and connected to a computer. An automated video tracking system recorded the position of the rat and calculated total distance moved and time spent in the central part of the field during the first 3 min. The first minutes appear to be the most sensitive period for detecting differences in the open field (Duncko et al., 2001; Westenbroek et al., 2003).

### **2.8.3 Object exploration**

In the open field apparatus familiar to the rat, three similar glass objects were placed equidistantly from the walls. An area of 5 cm around each object was defined as the zone of exploration. The rat was placed in the central zone and allowed to explore for 12 min. Total time spent in exploration of the objects during the first 3 min was measured together with the number of contacts made with the objects.

### **2.8.4 Acoustic startle and pre-pulse inhibition**

The startle response was measured in a chamber (SR-LAB Startle Response System, San Diego Instruments) with constant background noise at 70 dB. The rat was placed in a transparent cylinder (20 cm long, 9 cm in diameter) and its gross body movements were recorded through a pressure-sensitive plate at the presentation of acoustic test stimuli (120 dB, 40 ms). The maximum response ( $V_{max}$ ) was calculated. The cylinder was thoroughly cleaned between each test with a 5% ethanol solution.

The startle response can be modulated by a presentation of a pre-pulse in close temporal proximity, where a pre-pulse of high intensity reduces the amplitude of the startle response (pre-pulse inhibition) (Koch, 1999). To measure the level of inhibition, each test lasted for 140 ms consisting of: one 20 ms pre-pulse (73, 75, 80 or 85 dB) followed by 80 ms intermission and one 40 ms 120 dB pulse. To calculate the level of inhibition (%) for each of the pre-pulse intensity,  $V_{max}$  response to a pre-pulse test was compared to the  $V_{max}$  startle response where a pre-pulse was not used.

## 2.9 Statistical analyses

All statistical analyses were performed using Statistica (version 8.0, 10.0 or 12.0, StatSoft, Inc.). Significance was accepted at  $p \leq 0.05$ . The homogeneity of variance was tested using Levene's test. For all analyses "group" was used as independent factor. The following analyses were used: t-test for independent samples (homeostatic sleep pressure); one-way analysis of variance (ANOVA) (corticosterone; sucrose preference; open field test parameters; startle response); ANOVA for repeated measures (sleep parameters, EEG power frequencies and homeostatic sleep pressure; linear and circadian rhythm parameters of body temperature, locomotor activity and heart rate; sucrose preference and sucrose intake); ANOVA with factorial design (pre-pulse inhibition; object exploration parameters; startle response); ANOVA for repeated measures with factorial design (sucrose preference; open field test parameters; startle response); Pearson's product-moment correlation coefficient (between: maternal care following reunion after maternal separations and reduction in body temperature; maternal care and pre-CMS levels of corticosterone).

All significant overall ANOVA effects were further tested with Fisher's least significant difference post hoc test. Cohen's  $d$  was used to estimate the effect sizes for between group ( $d = (M1 - M2) / SD_{pooled}$ ) and within group ( $d = (M1 - M2) / (\sqrt{((SD1^2 + SD2^2) / 2)})^{-1}$ ) comparisons. An effect size of 0.2 is considered to be small, around 0.5 to be a medium effect and 0.8 and above, a large effect (Cohen, 1992).

### 3. Summary of results

#### Paper I

#### Early and later life stress alter brain activity and sleep in rats

This paper investigated sleep and EEG after exposure to long and brief maternal separations, and in combination with CMS. As an effect of early life condition, adult LMS offspring showed lower EEG power in the FF derivation during both sleep (Figure 4) and wakefulness, compared to BMS offspring. The quality of SWS differed as a consequence of maternal separation. LMS offspring showed more deep SWS (SWS2) but had lower delta power in the FF EEG and a slower reduction of the sleep pressure (low-range delta power, 0.5-2.5 Hz) compared to BMS offspring.

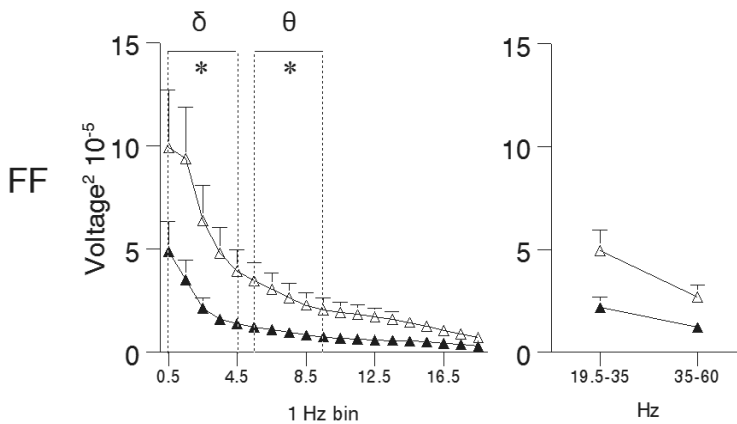


Figure 4. Frontal-frontal (FF) electroencephalogram power during slow wave sleep in adult long (filled triangles) and brief (white triangles) maternally separated offspring pre exposure to chronic mild stress.  $\delta$ =delta and  $\theta$ =theta frequency, \* $p < 0.01$ .

Exposure to CMS led to similar reductions in EEG power during sleep and wakefulness in both groups. A lower FF EEG power was still present in LMS compared to BMS offspring. CMS affected sleep differently in the two groups. LMS offspring showed longer total sleep time, more episodes of REM sleep and higher percentage of NREM episodes ending in REM sleep compared to BMS offspring.

Other sleep parameters were similar between groups, as well as reduction of the sleep pressure which was slower in both BMS and LMS offspring after CMS.

## Paper II

### Hypothermia after chronic mild stress exposure in rats with a history of postnatal maternal separations

Here, circadian rhythmicity of body temperature, locomotor activity and heart rate after exposure to long and brief maternal separations, and in combination with CMS were investigated. As an effect of early life condition, adult LMS offspring showed a delayed body temperature acrophase compared to BMS offspring. Investigation of all other parameters showed that circadian rhythms of body temperature, locomotor activity and heart rate were similar between adult LMS and BMS offspring.

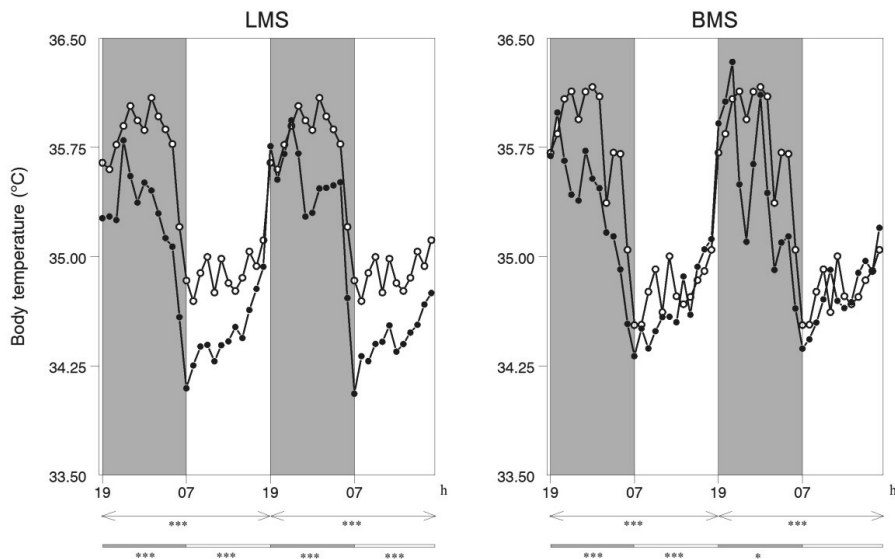


Figure 5. Body temperature in long (LMS) and brief maternally separated (BMS) offspring, 48 h pre (white circles) and post exposure to chronic mild stress (filled circles). Shaded area represents dark (active) phase. \* $p < 0.05$ , \*\*\* $p < 0.001$

Circadian rhythmicity was disturbed during the CMS exposure. After CMS exposure, a reduction of mean body temperature was observed in both groups (Figure 5). The hypothermia was stronger and longer lasting in LMS compared to BMS offspring. The heart rate of the LMS offspring was descriptively lower than the heart rate of the BMS group (d ranging from -0.6 to -0.8). Locomotor activity was not altered differently in the two groups. Both LMS and BMS offspring showed reduced preference for sucrose.

The degree of mothers' active care was reflected in the degree of hypothermia in LMS offspring (figure 6). More active nursing following maternal separation moderated the degree of hypothermia. More active nursing was also associated with higher levels of corticosterone before CMS exposure (figure 5). These associations were not observed for the BMS offspring.

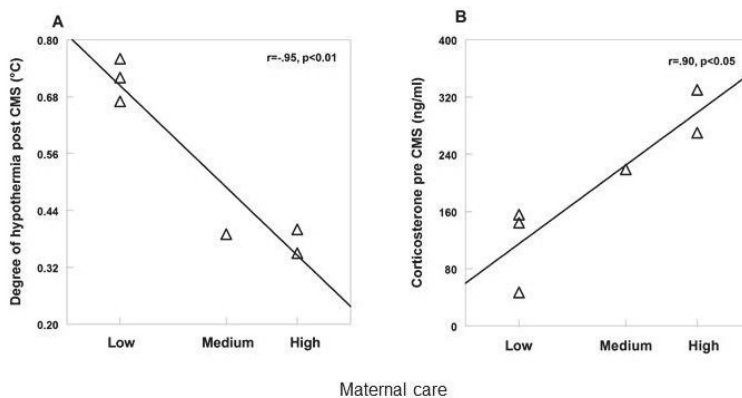


Figure 6. Active maternal care at reunion after long maternal separations associated with (A) degree of hypothermia induced by chronic mild stress (CMS) exposure and (B) levels of corticosterone pre CMS.

### Paper III

#### **Toughening up effect of adult chronic mild stress in rats experiencing the constant presence of the mother early in life**

This paper concerned three early life conditions (LMS, BMS and NH) and the interaction with CMS exposure. As a consequence of early life condition, there were

no differences in sucrose preference and activity in open field between LMS, BMS and NH offspring. NH and LMS offspring performed poorer in the object exploration task than BMS offspring. NH also showed lower pre-pulse inhibition than LMS and BMS offspring, and LMS displayed higher basal level of corticosterone than BMS offspring.

Four weeks of CMS induced an anhedonic-like state in all offspring. Upon CMS exposure, NH offspring increased their object exploration (Figure 7), habituated to acoustic startle and increased pre-pulse inhibition.

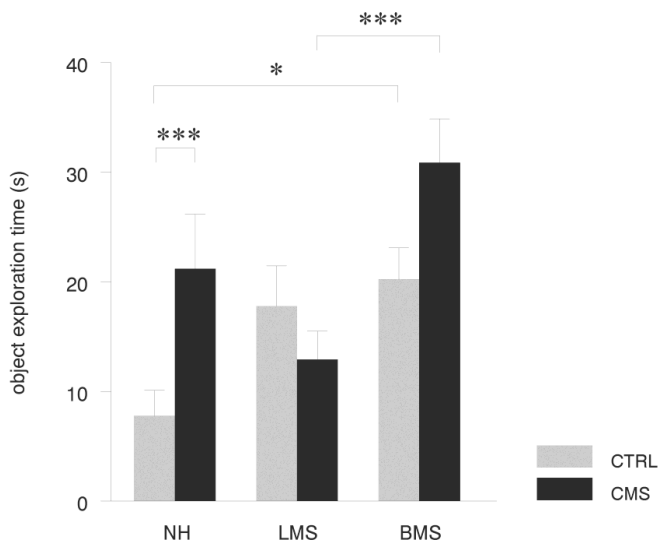


Figure 7. Object exploration in three different early life conditions exposed to chronic mild stress (CMS) or control condition (CTRL, see section 2.4 for more details). \* $p < 0.05$ , \*\*\* $p < 0.001$

CMS reduced object exploration in LMS compared to BMS offspring (Figure 7) and LMS offspring displayed a lack of habituation in the acoustic startle test. An initially high level of corticosterone was not further elevated by CMS, in contrast to NH and BMS offspring. Upon CMS exposure, BMS offspring increased object exploration (Figure 7).



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## 4. Discussion

### 4.1 Consequences of different early life conditions

#### 4.1.1 Brain activity measured by EEG and sleep

##### *EEG power*

In the large body of data from both clinical and preclinical studies on early life adverse events, measures of brain activity have not received great attention. A few clinical studies have described alterations in brain activity associated with early life adverse events (McFarlane et al., 2005; Tarullo et al., 2011). In the present study (Paper I), brain activity was investigated by means of EEG power, a heretofore unexplored area in studies on maternally separated rats. The results revealed a sleep-wake nonspecific reduction in adult brain activity at the frontal EEG derivation in LMS compared to BMS offspring. This effect of early life condition persisted upon exposure to chronic stress. CMS reduced brain activity in both groups, with LMS offspring showing lower EEG power compared to BMS. This finding was consistent across frequencies, during both sleep and wakefulness, particularly in the low oscillating frequencies typical of SWS, as well as the higher frequencies characteristic of REM sleep and wakefulness. The results suggest that maternal separations, longer than considered natural, may cause deviation from the normal development of EEG rhythms in rats. A decrease in the EEG power may reflect a decrease in both amplitude and/or number of the waves (Armitage, 1995). The present study did not investigate possible underlying neuronal changes that may be responsible for the dampened EEG power. However, it may be reasonable that altered EEG power in the present study reflects neuronal changes previously associated with LMS (Plotsky & Meaney, 1993; Hsu et al., 2003; Andersen & Teicher, 2004; Mirescu et al., 2004; Card et al., 2005; Aisa et al., 2007; Leventopoulos et al., 2007; Aisa et al., 2009b). Lower EEG power in LMS offspring was found mainly in the frontal derivation compared to BMS offspring, which may reflect possible changes in frontal cortical structures in particular, such as reduced volume, decreased number of synapses or altered monoaminergic innervation (Aksic et al., 2013; Chocyk et al.,

2013; Ohta et al., 2013), and possibly affect cognitive functions such as social interaction, habituation and spatial learning, associated with the frontal cortical structures (Uylings et al., 2003).

In humans, normal developmental changes in the brain structure are reflected in EEG power during both sleep and wakefulness (Somsen et al., 1997; Martinovic et al., 1998; Jenni & Carskadon, 2004). Findings of reduced EEG power of the higher frequencies during wakefulness in human children and adults with a history of early life adverse events (McFarlane et al., 2005; Tarullo et al., 2011) are thought to reflect differences in brain maturation (Marshall et al., 2004; Tarullo et al., 2011). Lowered power of the wakefulness-characteristic EEG frequencies in LMS offspring may parallel these findings. In humans, a history of adverse childhood events has also been associated with an altered posterior cortical processing upon exposure to unpleasant stimuli (Weber et al., 2009). The present study may reflect this, as the EEG power of the gamma frequency in the FP derivation was lower in LMS compared to BMS offspring after exposure to CMS.

### *Sleep homeostasis*

A further finding in Paper I was that LMS during early life may affect sleep homeostasis. This is the first study to report results on this topic, as sleep homeostasis has not yet been addressed in clinical or preclinical studies concerning adverse early life events. LMS offspring showed a slower homeostatic reduction in sleep pressure compared to BMS offspring as measured by low range delta (0.5-2.4 Hz) power during SWS. An altered homeostatic regulation has been proposed as one of the underlying mechanisms of disturbed sleep in depression, a hypothesis which was derived from a two-process (circadian and homeostatic) model of sleep regulation (Borbely et al., 1984). Hence, the present findings are consistent with a slower homeostatic reduction of slow wave activity across time spent in NREM sleep in depressed patients compared to healthy controls (Hoffmann et al., 2000; Armitage et al., 2001). Underlying mechanisms remain to be investigated. However, a recent computer model of activity in the homeostatic network suggests that the quantity of EEG power in the delta band is directly related to synaptic strength (Esser et al.,

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2007). It has been suggested that the level of slow wave activity during sleep may be a function of synaptic efficacy on network synchronization (Tononi & Cirelli, 2006). Accordingly, early life adverse events may influence the brain in terms of processing high-amplitude slow waves sufficiently, and weakening the sleep homeostasis through either impaired synaptic activation during wakefulness and/or reduced synaptic downscaling during sleep. Another possible explanation of decreased slow wave activity is disturbed sleep. Sleep disturbances such as insomnia, increased nightly awakenings and restless sleep are frequently reported in humans with a history of early life adverse events (Bader et al., 2007; Csoka et al., 2011; Steine et al., 2012a). One study in rats found increased wakefulness and reduced total sleep time after LMS (Feng et al., 2007). However, the present study did not find more fragmented sleep or increased wakefulness in the LMS offspring compared to the BMS. On the contrary, LMS offspring spent more time in SWS2 compared to BMS offspring. However, the finding concerning reduced delta power may reflect a reduced quality of SWS in LMS offspring. Thus, the results in the present study suggest that early life adverse events may impair homeostatic sleep regulation by reducing the quality of slow waves. Impaired homeostatic sleep regulation in human depression is reflected in the reduced slow wave activity (Borbely et al., 1984).

### *Sleep changes*

Adult LMS offspring showed more SWS2 compared to BMS offspring, however the reduced EEG power in the delta frequency suggests a diminished quality of SWS in LMS offspring.

Exposure to CMS reduced sleep quality and sleep quantity in both LMS and BMS offspring, alterations that have previously been associated with CMS exposure in animal facility reared rats (Grønli et al., 2004; Grønli et al., 2012). Even though CMS flattened the curve of homeostatic sleep pressure in both LMS and BMS offspring, sleep quality and quantity were affected differently in the two groups. Compared to BMS offspring, LMS offspring showed longer total sleep time, more REM sleep episodes and a higher percentage of NREM episodes ending in REM sleep. Typical REM sleep disturbances associated with human depression are increased amount of

REM sleep and/or reduced REM sleep latency (Peterson & Benca, 2006). The present study did not find higher amount of REM sleep, and as the animals were not disturbed to obtain a continuous telemetric recording, the REM sleep latency could not be investigated. However, the present findings concerning REM sleep episodes after CMS may indicate an increased pressure for REM sleep in LMS offspring after exposure to chronic stress. Similar REM sleep alterations have been described in a model of depression with highly stress reactive mice (Fenzl et al., 2011). Only one previous study combining maternal separations and stress exposure in adulthood examined sleep. Here, 1 h exposure to cold stress increased SWS and REM sleep in both BMS and LMS offspring (Tiba et al., 2004). The topic of combining early and later life stress deserves further investigation on sleep changes, particularly in the view of frequent sleep disturbances after early adverse events and their potential aetiological role in human depression.

#### **4.1.2 Circadian rhythms**

A small number of preclinical (Ackerman et al., 1978; Yoshihara et al., 2005; Yee et al., 2011; Feng et al., 2012; Trombini et al., 2012) and clinical studies (Glod et al., 1997; Bader et al., 2007) have investigated circadian rhythms after early life adverse events, providing only limited results on this topic. The present study investigated circadian rhythms of body temperature, locomotor activity and heart rate in adult LMS and BMS offspring, and effects of CMS exposure (Paper II).

##### ***Body temperature***

Investigation of the circadian rhythmicity in adult LMS and BMS offspring revealed a different timing of the body temperature circadian peak, occurring later in the LMS offspring. Based on all other parameters, LMS and BMS offspring demonstrated similar circadian rhythms of body temperature. In a previous study, stress during the juvenile period in rats reduced circadian amplitude of body temperature measured in adulthood (Yee et al., 2011). No clinical reports exist on early life adverse events and alterations in body temperature. However, studies of depressed patients have suggested circadian alterations in terms of both advanced and delayed circadian phase

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(Avery et al., 1982; Hasler et al., 2010). A delayed acrophase observed after LMS may thus indicate possible changes in the adult circadian regulation.

CMS exposure resulted in a lower body temperature in both groups of offspring. However, LMS offspring showed markedly stronger and longer lasting hypothermia. While body temperature in BMS offspring returned to pre-CMS levels, LMS offspring displayed hypothermia that persisted during the whole 48 h recording period. This suggests that BMS might provide stress resilience as measured here by a moderate thermoregulatory response, while adult thermoregulatory processes in LMS offspring were more strongly challenged by CMS, resulting in stronger body temperature alteration.

In a previous study, CMS exposure induced acute hyperthermia in the inactive phase in rats with no history of maternal separations (Ushijima et al., 2006), whereas CMS exposure in the present study led to hypothermia. The reason for this discrepancy is not clear. Maternal care plays an important role in the development of temperature regulation and circadian rhythmicity in the offspring (Conklin & Heggenes, 1971; Ohta et al., 2002). Although further investigation is needed, LMS or low maternal care during early PNDs could affect the developing circadian system. One study associated a reduced weight of the pineal gland with exposure to LMS (Feng et al., 2012). In addition, changes in the melatonin circadian regulation have been found in non-human primates reared in the absence of the mother (Rawashdeh & Dubocovich, 2014). The present study identified an enduring impact of LMS on the thermoregulatory response, a result conceptually similar to the few existing reports investigating early life stress and circadian parameters in rats. Weaning earlier than considered natural (i.e. before PND 22) was associated with strong hypothermia upon a 24 h restraint stress in adulthood (Ackerman et al., 1978). In another study, adult rats with a history of juvenile stress displayed chronic hypothermia and a stronger thermoregulatory response upon exposure to an acute stressor, compared to controls (Yee et al., 2011).

Several brain regions and neurotransmitter systems, such as the serotonergic system, play a role in central thermoregulation (Morrison et al., 2008). In depression, hyperthermia is one of the observed circadian abnormalities (Avery et al., 1982) which may be associated with alterations in serotonergic activity involved in temperature regulation (Rausch et al., 2006). Changes in the rat serotonergic system have been described after LMS (Benekareddy et al., 2010) and after CMS (Grønli et al., 2007). Such alterations may be one of the underlying mechanisms for hypothermia observed in LMS offspring in the present study.

### *Locomotor activity and heart rate*

LMS and BMS offspring showed similar circadian rhythmicity of locomotor activity in adulthood (Paper II) reflecting similar observations in a previous study (Feng et al., 2012). Moreover, exposure to CMS induced several similar effects on locomotor activity in both groups of offspring, such as increased activity during the inactive phase. The latter finding, although short term, may parallel increased nocturnal activity in adult humans with a history of adverse childhood events (Bader et al., 2007). Previously, CMS reduced activity in the active phase in rats without a history of maternal separations (Gorka et al., 1996), a finding observed in the present study for the BMS, but not LMS, offspring.

Circadian rhythmicity of heart rate was similar between LMS and BMS offspring in adulthood. Yet, a descriptively lower mean heart rate was observed in LMS offspring. A previous comparison between LMS and NH offspring showed no differences (Trombini et al., 2012), whereas reduced heart rate has been described in infant non-human primates during maternal separation (Reite et al., 1982). Exposure to CMS in the present study had a similar effect on heart rate in both groups, decreasing all mean values. Yet, the descriptively lower heart rate in LMS compared to BMS offspring persisted. Adult humans with a history of severe childhood abuse vs healthy controls showed enhanced acute stress responsivity in terms of increased heart rate (Heim et al., 2000b). Taken together, the results in the present study do not indicate different responses in circadian rhythms measured by locomotor activity and heart rate related to different early life conditions.

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### *Circadian rhythmicity during CMS*

Circadian patterns in body temperature, locomotor activity and heart rate were also monitored during the CMS exposure, aiming to explore the impact of individual stressors contributing to the changes observed after CMS. Descriptively, CMS disrupted normal circadian rhythmicity in both groups. Upon exposure to stressors “wet bedding”, “food deprivation” and “water deprivation”, the LMS offspring showed lower body temperature and a descriptively lower heart rate compared to BMS offspring. Food and water deprivation during the rats’ active phase stimulate food and water consumption during their inactive phase. As a consequence, disturbed body temperature, activity and heart rate, are likely to prolong the effect of deprivation stressors even after their termination. This is in agreement with a previous study, although not ruling out the potential influence of locomotor activity on disturbed body temperature and heart rate (Nielsen, 2001). In the present study, lower body temperature and heart rate in LMS offspring were not reflected in locomotor inactivity. Another weekly stressor, 36 h continuous light exposure, may have a direct impact on the circadian parameters given that light is the strongest zeitgeber. However, results from previous studies indicate that circadian rhythmicity of body temperature is disturbed only after continuous light exposure longer than 36 h (Honma & Hiroshige, 1978; Depres-Brummer et al., 1995). The altered body temperature found in the present study is likely an outcome of the CMS protocol as a whole, involving the unpredictability, variety, frequency and the moderate intensity of stressors. Notably, the alteration was stronger in LMS compared to BMS offspring.

#### **4.1.3 Corticosterone**

In Papers II and III, levels of corticosterone were used as an indicator of HPA axis activity in LMS, BMS and NH offspring. In Paper III, the three early life conditions showed different effects on adulthood levels of corticosterone, with LMS offspring showing higher levels compared to BMS. In Paper II, concerning a subgroup of LMS and BMS offspring, LMS showed descriptively but not significantly higher levels of corticosterone. These results are in line with some previous reports, whereas other studies did not find differences in basal levels of corticosterone comparing different

early life conditions (Plotsky & Meaney, 1993; Pryce et al., 2001; Lippmann et al., 2007; Uchida et al., 2010; Oines et al., 2012). Increased levels of corticosterone may be attributed to alterations in negative feedback regulation of the HPA axis in adult LMS compared to BMS offspring (Plotsky & Meaney, 1993; Francis et al., 2002; Ladd et al., 2004). In the present study, corticosterone was similar in LMS and NH offspring, supporting several previous reports (Wigger & Neumann, 1999; Sloten et al., 2006; Renard et al., 2007). Such an outcome may also reflect altered HPA axis regulation in NH offspring such as in increased levels of hypothalamic CRF mRNA (Plotsky & Meaney, 1993).

Upon CMS exposure a decrease in basal corticosterone in LMS offspring compared to BMS and NH offspring was expected. Results show that initially high level of corticosterone in LMS offspring was not further elevated by CMS, in contrast to NH and BMS offspring. In an earlier study comparing LMS and AFR condition, Renard and colleagues found no difference in basal level of corticosterone before exposure to chronic variable stress, but found LMS' corticosterone to lower after chronic stress exposure (Renard et al., 2007). Another study reported that chronic variable stress exposure in LMS offspring decreases their responsivity to acute stress (Ladd et al., 2005). Based on these observations it has been proposed that chronic stress may dampen the pituitary-adrenal response in LMS offspring by enhancing transcription of glucocorticoid receptor in the cortex (Ladd et al., 2005; Renard et al., 2010). Our results support this notion.

Taken together, different basal levels of corticosterone and different HPA axis regulation may explain different responsivity to adult chronic stress exposure in the three early life conditions. This reflects the importance of early life events for stress reactivity in adulthood. HPA axis alterations as a consequence of early life adverse events are closely associated with increased risk for psychiatric disorders in humans, particularly after additional exposure to stress later in life (Heim et al., 2008).

Whereas data on experimental exposure to chronic stress in humans are limited, studies addressing acute stress responsivity in adults humans with a history of



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childhood abuse have indicated alterations in HPA axis, and reflect the findings from preclinical studies (Heim et al., 2001; Heim et al., 2002).

#### **4.1.4 Affective-like behaviour**

One of the aims of the present study was to investigate whether different early life conditions would result in different affective-like behaviour in adulthood, as indicated earlier in the literature (Huot et al., 2001; Francis et al., 2002; Lambasenas et al., 2009; Hui et al., 2011).

The three early life conditions showed similar sucrose preference in adulthood (Paper III). Also, no differences in sucrose preference were observed between subgroups of LMS and BMS offspring in Paper II. Thus, no anhedonic-like state was revealed in the present study. Previous studies show inconsistent results as both decreases, increases or no differences in sucrose preference have been reported in LMS offspring (Shalev & Kafkafi, 2002; Mourlon et al., 2010; Uchida et al., 2010; Hui et al., 2011; Oines et al., 2012; Zhang et al., 2013b). Further, in the present study, adult LMS, BMS and NH offspring showed no difference in spontaneous activity (central and total distance moved) in the open field. From earlier studies, total distance moved did not show to be affected by the early life environment (Lehmann et al., 2000; Arnold & Siviyy, 2002; Vivinetto et al., 2013). Low central activity in the open field test may indicate an increase in anxiety-like behaviour, an outcome previous studies have reported in LMS compared to BMS or NH offspring (Francis et al., 2002; Diehl et al., 2007), as well as LMS and NH compared to BMS offspring (Caldji et al., 2000). Acoustic startle responses were not different between the three early life conditions, confirming no anxiety-like behaviour in any offspring. The results are in line with earlier reports concerning LMS and NH offspring (Caldji et al., 2000; de Jongh et al., 2005), while other studies have described increased startle response in LMS compared to BMS and NH offspring (Kalinichev et al., 2002; Lippmann et al., 2007). Some studies report enhanced startle response in adult NH offspring (Caldji et al., 2000; Pryce et al., 2001).

Taken together, the behavioural measures in the present study, sucrose preference, activity in the open field and acoustic startle, did not reveal affective-like behaviour in adult LMS and NH compared to BMS offspring as a consequence of early life condition. Supporting the results from open field assessment, a 24 h measurement of locomotor activity in their home-cage in Paper II did not reveal differences between LMS and BMS offspring. On the other hand, LMS offspring showed a reduction in EEG power within the delta frequency range, altered sleep homeostasis (Paper I) as well as delayed body temperature acrophase (Paper II), findings which may suggest depression-like changes in LMS offspring. In support of this, increased levels of corticosterone in adult LMS offspring (Paper III) are in line with increased levels of cortisol associated with human affective disorders (Arborelius et al., 1999; Heim et al., 2008).

Affective-like behaviour was also investigated in the three early life conditions upon exposure to CMS. In rats, CMS exposure typically induces an anhedonic-like state reflected by reduced sucrose preference, an observation useful when considering the CMS paradigm as a model of stress related depression (Willner, 2005). Reduced sucrose preference following CMS exposure in the present study (Papers II and III) indicates an anhedonic-like state in all offspring, confirming the effect of the CMS paradigm. This is in contrast to one previous study describing stronger anhedonic-like state in NH compared to BMS offspring upon exposure to CMS (Bouffleur et al., 2013). That study did not include an LMS group. In the present study, all offspring showed a similar activity in the open field test. Previously, chronic variable stress resulted in lower open field activity in LMS compared to AFR offspring (Renard et al., 2007) whereas our results are in line with no differences in open field activity between LMS and AFR offspring after repeated exposure to footshock (Hulshof et al., 2011). On the other hand, in the present study CMS exposure enhanced habituation of the acoustic startle response in BMS and NH offspring, whereas LMS offspring failed to show habituation. As such, the results may indicate more vulnerability to stressful life events in adulthood in LMS offspring.

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In summary, the present results on sucrose preference, activity in the open field and acoustic startle do not confirm increased affective-like behaviour in LMS and NH compared to BMS offspring upon exposure to later life stress. A possible indication of affective-like changes in LMS offspring may be in the increased pressure for REM sleep observed after CMS (investigated in a subset of LMS and BMS offspring, Paper I).

#### **4.1.5 Cognitive performance**

Adverse early life events in humans may be associated with impaired cognitive functioning later in life (Hedges & Woon, 2010). In animal models, adverse early life events have also been associated with cognitive impairment, such as altered performance in a water maze and object exploration task after LMS in rats (Aisa et al., 2007; Daniels et al., 2009). In the present study in the object exploration task (Paper III) adult LMS offspring showed fewer contacts with objects compared to BMS offspring, but similar object exploration compared to NH offspring. NH offspring spent less time in object exploration compared to BMS offspring. These results confirm the hypothesis on reduced cognitive performance in LMS and NH compared to BMS offspring, a finding also reported in other laboratories (Mourlon et al., 2010; Vivinetto et al., 2013). Also, NH offspring showed a reduced pre-pulse inhibition compared to LMS and BMS offspring, in contrast to previous reports on similar inhibition in the different early life conditions (Lehmann et al., 2000; Pryce et al., 2001). Together, the present results suggest an impaired cognitive performance in LMS and NH offspring.

CMS exposure increased object exploration in BMS offspring and improved their habituation to acoustic startle. A previous study investigating cognitive performance after later life stress found reduced performance in a novel object task in both NH and LMS offspring upon exposure to repeated footshock (Hulshof et al., 2011). In the present study, exposure to CMS had different effects on cognitive performance in these offspring. LMS offspring showed a reduction in object exploration compared to BMS offspring. Remarkably, NH offspring displayed more object exploration upon

exposure to CMS. Moreover, an impaired pre-pulse inhibition was improved in NH offspring exposed to CMS. Pre-pulse inhibition represents a behavioural neurobiological measure, a measure of sensorimotor gating. Whether pre-pulse inhibition may reflect higher mechanisms is questionable (Koch, 1999). Certainly, data on increased pre-pulse inhibition in NH offspring support the results on increased cognitive performance in the object exploration task when exposed to CMS.

The present study investigated cognitive performance in object exploration task (Paper III) and brain activity measured by EEG power in LMS and BMS offspring (Paper I). A link between brain activity and cognitive performance in animal models of affective disorders has not received much (if any) attention. Based on the present study, future investigation could be to explore a possible association between altered brain activity and cognitive performance. Given the differences between BMS and LMS offspring, whether higher object exploration would be associated with higher EEG power is a hypothesis remaining to be investigated.

#### **4.1.6 Moderating factors**

##### **Active maternal care**

Human studies suggest that effects of early life adverse life events may be moderated by other experiences, such as perceived parental care, having a stable caregiver or through perceived social support (Kaufman & Henrich, 2000; Collishaw et al., 2007; Steine et al., 2012b). Variations in the HPA axis response to a psychosocial challenge in adulthood may also be related to perceived amount of maternal care during early life (Engert et al., 2010). In rats, naturally occurring high levels of active maternal care may have long-lasting positive effects on the behaviour of the offspring and their stress response in adulthood, effects that are associated with the stronger negative feedback regulation of the HPA axis (Liu et al., 1997; Francis et al., 1999). Offspring of the mothers that display high amount of licking and grooming show signs of increased synaptogenesis as well as improved cognitive performance in adulthood (Liu et al., 2000b). In the present study, the mothers in the LMS condition showed different levels of maternal care following reunion during PND 2-7. One of the aims

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in Paper II was to explore if levels of active maternal care were associated with changes in the circadian rhythmicity induced by a combination of early life condition and adulthood CMS exposure, and with levels of corticosterone pre CMS. Those offspring receiving more active maternal care showed less CMS-provoked hypothermia in adulthood. A stronger hypothermia was present in those offspring experiencing lower levels of active maternal care. Due to a low number of mothers (n=3) and offspring (n=6) included in the analysis, definite conclusions are limited. Yet, the results may add to the literature suggesting that experiencing higher levels of active maternal care may moderate the enduring impact of LMS. The results support the notion that individual variability in the vulnerability to later life stress may be associated with quality of maternal care (Henningsson et al., 2012).

Receiving less active maternal care after reunion was associated with lower corticosterone level pre CMS in LMS offspring. This may reflect the studies in both humans and non-human primates associating low levels of cortisol with low parental care (Gunnar & Vazquez, 2001; Engert et al., 2011). Hypocortisolism is thought to be a result of the prolonged overactive HPA axis which may cause long-lasting changes in its negative feedback regulation (Heim et al., 2000a; Fries et al., 2005). In addition to its main role in HPA axis regulation, the paraventricular nucleus of the hypothalamus also contains thermosensitive neurons which mediate autonomic mechanisms of thermoregulation (Nunn et al., 2011). Although possible changes in the upper parts of the HPA axis were not investigated in the present study, the CMS-induced hypothermia might be partly mediated through changes in HPA axis activity, where active maternal care appeared to be a moderating factor.

### **Brief maternal separations**

In the course of interactions between the rat mother and the offspring, normal behaviour involves not only feeding and nursing of the offspring but also short regular periods away (e.g. 20-30 min). This natural behaviour has also been demonstrated in the laboratory setting, where the design of the housing conditions allows for such (Grota & Ader, 1969; Jans & Woodside, 1990). Hence, experimental exposure to BMS resembles naturally occurring phenomena. In the offspring,

exposure to BMS condition has been associated with reduced startle response and increased open field exploration and cognitive performance (Meaney et al., 1988; Caldji et al., 2000). Upon a physiological or psychological challenge in adulthood, the BMS offspring usually display less activation of the HPA axis compared to LMS or NH offspring (Levine, 1957; Caldji et al., 2000). The results on BMS offspring in the present study follow these findings, as in higher EEG power, normal reduction in sleep pressure (sleep homeostasis), and lower levels of corticosterone. In line with lower stress responsivity in BMS offspring, fewer REM sleep episodes, less and shorter lasting hypothermia, as well as better performance in the object exploration task were observed after CMS. Together, the findings add to the literature showing that brief separations from the mother during early life may have long-lasting positive effects for the offspring.

### **Chronic mild stressors**

Housing in standard animal facility caging system (such as the IVC system used in this study) does not allow for the natural maternal behaviour of short periods away. In view of this, early life condition in terms of the constant presence of the mother may not be optimal. The offspring may lack the positive effect of short-term triggering of the stress-regulating mechanisms during their developmental period and the NH mothers' active maternal care may not come naturally since the environment is missing natural triggers to evoke maternal response. Accordingly, NH as an early life condition without brief periods of maternal absence may "deprive" the offspring of stimuli that are important for the normal development of the stress response. Indeed, these offspring can display phenotypes similar to the LMS offspring. The results in the present study indicate that early life condition with constant presence of the mother as well as experiencing LMS may induce negative consequences in adulthood observed as poor performance in the object exploration and reduced pre-pulse inhibition. Several studies have described similar effects on affective-like behaviours and cognitive performance in adult NH and LMS offspring (de Jongh et al., 2005; Madruga et al., 2006; Mourlon et al., 2010; Vivinetto et al., 2013). Remarkably though, results on behavioural tests indicate that adult CMS exposure may produce a positive effect in NH offspring. After being exposed to daily mild stressors for four

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weeks, NH offspring increased their object exploration, habituated to acoustic startle and increased pre-pulse inhibition. Thus, lacking adequate stimuli during early life, a “toughening up” effect in these offspring may be achieved through exposure to mild daily hassles during their adulthood.

#### **4.1.7 Summary of study outcomes**

The present study describes effects of different early life conditions (LMS, BMS or NH) on adulthood functioning and different effects of exposure to chronic stressors in adulthood as a consequence of early life condition. EEG power indicated a difference in brain activity between adult LMS and BMS offspring. LMS offspring showed lower EEG power, as well as poorer quality of SWS and weakened sleep homeostasis. CMS lowered EEG power in both groups of offspring; however the lowered EEG power was still present in LMS offspring. While no robust differences were detected on their circadian rhythmicity as a consequence of early life condition, the impact of CMS exposure on body temperature reduction was stronger in LMS offspring compared to BMS. Here, the active maternal care during early life seemed to be important and reduced the impact of CMS in LMS offspring.

LMS offspring showed highest levels of corticosterone, which were not further elevated by CMS exposure in contrast to BMS and NH offspring, a result which may reflect different regulation of the HPA axis as described in earlier studies. Whereas EEG findings may reflect some affective-like changes in LMS offspring, no differences on affective-like behaviour were found between adult LMS, BMS and NH offspring either as a consequence of early life conditions or in combination with CMS exposure.

Both LMS and NH offspring showed an indication of reduced cognitive performance compared to BMS, which reflects the adverse consequences of both long maternal separations and condition with the constant presence of the mother. Upon CMS exposure, reduced cognitive performance was still present in LMS offspring compared to BMS, whereas CMS exposure improved cognitive performance in NH

offspring. These results suggest a toughening up effects in NH offspring by exposure to mild daily hassles.

Overall, the results indicate that exposure to LMS during early life may have adverse consequences for brain functioning as reflected in measures of brain activity and cognitive performance. Brain activity was not assessed in the NH offspring, while the results on cognitive performance suggest adverse consequences of this early life condition. Exposure to CMS affected brain activity in both LMS and BMS offspring while it had different impact on their thermoregulatory response and CMS affected cognitive performance differently in the three early life conditions. Overall, the present findings reflect the notion that different experiences and hence different developmental conditions during early life may have consequences for adulthood brain functioning.

## 4.2 Methodological considerations, strengths and limitations of the study

### 4.2.1 Surgical procedure

Results in Papers I and II were acquired from animals implanted with telemetric devices. It is known that surgery can affect many physiological functions, including both sleep quality and circadian rhythmicity (Tang et al., 2007; Moscardo & Rostello, 2010). Those data were based on surgical procedures similar to those used in this study. Both the amount of NREM sleep and REM sleep as well as power of the characteristic EEG frequencies stabilize within the first postoperative week, whereas locomotor activity parameters show gradual recovery in circadian rhythmicity within a period of two weeks (Tang et al., 2007; Moscardo & Rostello, 2010). In both Papers I and II, a minimum of two weeks of postoperative recovery was allowed. We did not record postoperative EEG or circadian rhythmicity. On the assumption that all parameters measured were stabilized prior to the assessment, and given that all animals were exposed to the same procedures, there is reason to believe that the differences detected between the groups in Papers I and II represent the effects of



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different early life conditions, i.e. LMS and BMS. Yet, given the possible differences in the stress regulating mechanisms as a consequence of LMS and BMS, surgical procedure as a physiological challenge may affect LMS and BMS offspring differently and influence the results.

Furthermore, LMS and BMS animals with implants were also included in Paper III. As a result LMS and BMS groups in Paper III consisted of both implanted and non-implanted animals. To control for the surgical procedure, all of the non-implanted animals also underwent sham surgery, involving exposure to the same pharmacological treatment including anaesthesia, analgesia and antibiotic treatment as well as handling. However, the surgical procedure itself was not as invasive as the one required for implanting the transmitters, reflected in the shorter time for postoperative recovery. As a consequence, depending on the type of surgery, the animals may have experienced different levels of physiological stress, which may have potential consequences for the outcomes measured in Paper III. This may particularly be important when the results in the LMS or BMS group are compared with the NH group in which none of the offspring were implanted with a telemetric device.

#### **4.2.2 Telemetry data**

The period for monitoring the circadian rhythms in the present study (Paper II) was short (48 h). Monitoring circadian rhythms of body temperature over a longer period of time could have revealed chronic alterations. However, the 48 h recording was still sufficient to detect different thermoregulatory responses in the two groups (LMS and BMS) upon exposure to CMS.

The lack of sleep, EEG and circadian rhythm assessment in the NH group is a limitation in the present study. Low number of transmitters available in the laboratory allowed only two groups to be compared on these parameters (Papers I and II). So far only a limited amount of data exists on sleep and circadian rhythmicity in NH offspring, showing similar circadian rhythmicity of heart rate compared to LMS offspring, and upon exposure to an acute stressor, reduced amount of SWS in contrast

to LMS and BMS offspring (Tiba et al., 2004; Trombini et al., 2012). Future studies investigating sleep, EEG and circadian rhythmicity should include all three early life conditions, particularly given the different effects of early life conditions and CMS exposure on behavioural measures (object exploration, pre-pulse inhibition and acoustic startle) reported in the present study.

### **4.2.3 Gender perspectives**

The evidence from epidemiological studies points toward a higher risk for development of mood and anxiety related disorders in women than in men. Twice as many women suffer from major depression, which may also be more persistent and show more comorbidity with other disorders (World Health Organization, 2014). While the reason for this is yet to be fully understood, one explanation may be a gender difference in the prevalence of early life adverse events. Moreover, such events may have more detrimental consequences for the HPA axis regulation in female than in male subjects (Weiss et al., 1999). Studies on the consequences of different early life conditions in rats that include both female and male subjects often report sex differences in adulthood behaviour and stress reactivity (Wigger & Neumann, 1999; Kalinichev et al., 2002; Eklund & Arborelius, 2006). More importantly, exposure to adulthood chronic variable stress has been shown to induce stronger alterations in behaviour and molecular markers of HPA axis activity in female compared to male rats (Renard et al., 2007; 2010). Also, a few studies have indicated a differential effect of early life conditions on sleep stage duration in male and female rats, predominantly upon exposure to an acute stressor in adulthood (Tiba et al., 2004; 2008). Despite this, the majority of preclinical studies are performed on male subjects only. This represents a limitation in the present study as well. Hence, the consequences of LMS for the adult brain activity and circadian rhythmicity delineated in the present study should be examined in female offspring. An ongoing study is investigating EEG, sleep and circadian rhythms in female offspring after different early life conditions.

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#### 4.2.4 Sample size

One limitation of this study was the small sample size, particularly in Papers I and II where only LMS and BMS offspring were used, and no control LMS and BMS animals included (due to the limited number of transmitters available). To estimate the impact of the data, Cohen's *d* effect sizes were used. Effect sizes objectively measure the importance of an effect, independently of the sample size. In the present data set, many group differences showed medium to large effect sizes, e.g. characteristic EEG frequencies for the different sleep stages and wakefulness (Paper I). Hence, despite the low *n*, effect sizes reflect that the significant differences between the groups in the study are relevant.

Moreover, such effect sizes were also observed for some of the non-significant results, e.g. sleep parameters (Paper I), mean heart rate values (Paper II), corticosterone and object exploration (Paper III). Reaching no statistically significant difference and thus rejecting the hypothesis on differences between the groups, small sample size may increase the risk of committing a type II error. Therefore results showing large effect size, albeit not reaching a statistical significance, should be reported. This is particularly important given that animal research demands working with the principles of "3 R's".

Whereas the use of wireless recording addressed both the refinement and the reduction aspect of the principles of "3 R's" (Russel & Burch, 1959), assesment of several different parameters also increased the number of statistical comparisons. Owing to the number of comparisons made, some of the variables were grouped to control for type I errors (e.g. circadian rhythm parameters: amplitude and acrophase).

The number of mothers used in the study was low. The LMS offspring in Paper II had three different mothers. Therefore, the conclusions with respect to the effects of maternal behaviour after reunion are limited. Nevertheless, although considered in a descriptive manner, the results follow the literature on moderating effects of active maternal care and reflect this important aspect of early life environment.

Despite several limitations, the present study revealed differences between early life conditions, and contributes to the literature adding results on EEG power and circadian rhythmicity, as well as addressing the important aspect of combination of early life conditions and adulthood chronic stress exposure.

### 4.3 Implications and future directions

The findings in Papers I and II add to the literature on the consequences of early life events, investigating EEG, sleep and circadian rhythms after early life events and chronic stress in adulthood. The study revealed alterations in EEG power and sleep homeostasis after LMS in rats. It is well established that sleep disturbances are associated with many psychiatric disorders in humans, and sleep disturbances may even be involved in the aetiology of psychiatric disorders such as major depression and anxiety. Sleep EEG abnormalities typically associated with depression have been found in humans with a high risk for depression (Friess et al., 2008). In line with this, findings on EEG alterations in the present study may represent markers for vulnerability to stress and psychiatric disorders after exposure to adverse early life events. This highlights the need for more studies investigating brain activity such as measured by EEG, in individuals with a history of early life adverse events. Identification of sleep and EEG abnormalities could lead to earlier interventions and prevent development of psychiatric disorders. The finding of altered sleep homeostasis as a consequence of LMS suggests that possibly altered mechanisms in the homeostatic network and processing of the slow oscillating waves should be further investigated. Studies comprising measures of the activity in subcortical areas involved in generation of slow oscillations may be useful for delineating the underlying mechanisms.

Importantly, the present study adds to the literature on moderating factors of early life events showing that the amount of maternal care after LMS may be protective upon later stress exposure. Positive effects of naturally occurring high levels of active maternal care have been associated with the strengthening of the HPA axis regulation (Liu et al., 1997; Francis et al., 1999). It has further been shown that high levels of

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active care may improve cognitive performance in adulthood (Liu et al., 2000b). Moreover, other means of environmental manipulations, such as enrichment, may reverse some of the effects of adverse early life events (Francis et al., 2002; Bredy et al., 2003). Thus, further focus should be given to moderating factors of adverse life events and the underlying mechanisms, in future studies.

Notably, the results on object exploration, pre-pulse inhibition and acoustic startle habituation in NH offspring suggest that being exposed to a variety of mild daily hassles in adulthood may yield a positive effect in these offspring on their cognitive performance. Hence, this topic deserves further investigation. Adding to this, it may be reasonable to examine consequences of NH in comparison to LMS and BMS, under housing conditions that allow for more natural maternal behaviour.

## 4.4 Conclusions

The present study combined the maternal separation model with exposure to later life chronic mild stress, as one approach with potentially high etiological and construct validity within the research on early-life manipulation.

The study investigated consequences of different early life conditions (LMS, BMS or NH) in adult rats on EEG, sleep, circadian rhythms, HPA axis activity, affective-like behaviour and cognitive performance, and investigated consequences of exposure to unpredictable stressors (CMS) in adulthood.

The present study confirmed that brief separations early in life provide a “toughening up” effect in adulthood. BMS offspring showed higher EEG power and lower basal corticosterone level compared to LMS offspring, best performance in the object exploration task and a stronger pre-pulse inhibition compared to NH offspring.

LMS induced a sleep-wake nonspecific reduction in frontal EEG power, altered sleep homeostasis and a different quality of SWS as compared to BMS. LMS and BMS offspring demonstrated similar diurnal rhythms of body temperature, locomotor activity and heart rate. LMS offspring showed highest levels of corticosterone and

reduced object exploration. No differences were found on affective-like behaviour between NH, LMS or BMS offspring. NH and LMS offspring performed poorer in the object exploration task than BMS offspring, and NH offspring displayed lower pre-pulse inhibition compared to both LMS and BMS offspring. Hence, the results confirm that different early life events may have a long-lasting effect on brain function, whereas no differences were found regarding circadian rhythmicity or affective-like behaviour as a consequence of different early life conditions.

The combinations of early life conditions and CMS exposure affected sleep differently in the two groups of maternally separated animals (LMS and BMS). LMS induced a latent thermoregulatory disturbance which was evident upon exposure to later life stress as a stronger hypothermia in LMS compared to BMS rats. Levels of corticosterone were affected differently between LMS, BMS and NH offspring. CMS exposure induced an anhedonic-like state in all offspring reflecting affective-like behaviour independent of the early life conditions. In LMS offspring, some indications of increased anxiety-like behaviour and reduced cognitive performance were observed.

The impact of LMS on the thermoregulatory response upon CMS exposure seemed to be moderated by maternal care following the reunions in these offspring. Also, the data may suggest that brief postnatal separations from the mother provide stress resilience in the thermoregulatory response, independent of maternal care.

Remarkably, results on behavioural tests indicate that adult exposure to chronic mild stressors mimicking daily hassles in humans may produce a positive effect in NH offspring. These offspring increased their object exploration, habituated to acoustic startle and increased pre-pulse inhibition after being exposed to CMS for four weeks. These results support the notion that effects of early life experiences may be moderated through other experiences such as improved caregiving and environmental stimuli.

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