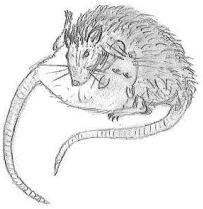
Consequences of social defeat stress for behaviour and sleep

Short-term and long-term assessments in rats



Anne Marie Kinn Rød

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Sketch of rats in a social defeat confrontation on the front page
is illustrated by Anne Marie Kinn Rød

Scientific environment

Working with the thesis, I have been employed as a PhD student at the Department of Biological and Medical Psychology, University of Bergen, Norway (2006-2011). The PhD grant was provided by the University of Bergen. I have also received a grant from the Norwegian Competence Center for Sleep Disorders, (January 2012).

The work for study I in this thesis was carried out at the Section of Physiology, Department of Biomedicine, University of Bergen, from July 2004 to August 2005, and resulted in my thesis for Master of Science. The thesis was edited to a paper which was published in 2008. The work for studies II and III was carried out at the Department of Biological and Medical Psychology, Research Group on Experimental and Clinical Stress and Sleep, University of Bergen, Norway. I was associated with The International Graduate School in Integrated Neuroscience at Department of Biological and Medical Psychology. My main supervisor was Robert Murison, and my co-supervisors were Anne Marita Milde, Janne Grønli and Håkan Sundberg, all with affiliation to the Department of Biological and Medical Psychology. Janne Grønli is also affiliated to the Norwegian Competence Center for Sleep Disorders (SOVno), Haukeland University Hospital. Anne Marita Milde is also affiliated to Resource center on Violence Traumatic stress and Suicide prevention (RVTS), Region West.

SOVno
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List of abbreviations

ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
ASD	acute stress disorder
ASR	acoustic startle response
CMS	chronic mild stress
CRH	corticotropin-releasing hormone
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders-4th edition
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders-4th edition-Text Revision
DSM-5	Diagnostic and Statistical Manual of Mental Disorders-5th edition
EEG	electroencephalogram
EMG	electromyogram
EOG	electrooculogram
EPM	elevated plus maze
FF	fronto-frontal
FP	fronto-parietal
GAD	generalized anxiety disorder
HPA	hypothalamo-pituitary-adrenocortical
ICD-10	International statistical classification of diseases and related health problems, 10th edition
IFS	inescapable foot shock
IVC	individually ventilated cages
LSD	least significant difference
MDD	Major Depressive Disorder
NREM	non rapid eye movement
OF	open field
PTSD	post-traumatic stress disorder

REM	rapid eye movement
sc	subcutaneous
S.E.M.	standard error of the mean
SD	social defeat
SDF	social defeat fighter
SDS	social defeat submissive
SWS	slow wave sleep
SWA	slow wave activity
Vmax	maximum response amplitude

Abstract

Social stressors play a major role in the pathogenesis of affective disorders like anxiety and depression. These disorders are associated with altered behaviour (i.e. locomotor activity, harm avoidance, startle response, anhedonia and sexual behaviour), sleep alterations and abnormalities in the stress response. The animal social defeat (SD) model is based on a natural conflict situation where a male intruder rat eventually subordinates itself to an unfamiliar territorial resident conspecific. The effects of defeat are studied in the intruder rat.

The main purpose was to study the face validity of the SD model for affective disorders by investigating short-term and long-term consequences of single and/or double exposure to SD on behaviour and sleep in rats. In particular, the intention was to evaluate if SD could reproduce the alterations in locomotor activity, harm avoidance, startle response, anhedonia, sexual behaviour, stress responses and sleep parallel to those observed in patients with affective disorders.

Social defeat induced low activity in the central sector of the open field (OF) (Paper I), indicating high harm avoidance which may reflect anxiety-like or depression-like behaviour. No short-term or long-term effects were seen on total locomotor activity in the OF (Papers I and II). Further, a lack of habituation to the OF across days was seen, which may reflect long-lasting heightened anxiety (Papers I and III). Overall, in the elevated plus maze (EPM) test, SD rats showed less total locomotor activity, less percentage time and less activity on the open arms, lasting up to 3 weeks after defeat (Paper II), possibly reflecting anxiety-like behaviours. High acoustic startle responses (ASR) were seen as a long-term effect of SD, probably reflecting an anxiety-like state (Paper II). A short-lasting reduced preference for sucrose was seen (Paper II), indicating an anhedonic state that may be interpreted as a transient anxiety-like symptom. Sexual behaviour was not affected (Paper I). As a group, SD rats did not show altered corticosterone responsiveness to OF exposure (Paper III).

The SD rats showed a short-term increase in duration of slow wave sleep (SWS) 2 and sleep fragmentation (Paper I). Overall, SD rats did not show long-term effects on sleep or EEG power (Paper III). The effects of SD on sleep may be

interpreted as anxiety, because they were short-lasting and the common sleep alterations seen in depression were not induced (e.g. reduced SWS2 and REM sleep alterations).

A secondary aim was to compare effects of SD to the effects of inescapable footshock (IFS) (Paper II). The two stressors induced a similar short-term effect on sucrose preference and similar long-term anxiety-like behaviours in the EPM test. Contrary to what was expected, SD rats showed the highest ASR, while IFS rats showed the lowest total activity in the OF test. The results may reflect fundamental differences between SD and IFS.

Another secondary aim was to explore the relationship between levels of corticosterone prior to SD or IFS stressor, and the different post-stressor behaviours (Paper II). Low pre-stress corticosterone level was expected to be associated with anxiety-like behaviours following stress. Overall, such a relationship was not found. Contrary to what was expected, the SD rats with high pre-stressor corticosterone level showed the greatest ASR, while IFS rats with low pre-stress corticosterone level did not show alterations in ASR. This further supports differences between the SD and the IFS stressor.

The final secondary aim was to investigate differences in effects of SD on behaviour and sleep in two subgroups of rats with different coping styles in the SD (Paper III). Contrary to what was expected, rats fighting back in the SD confrontation showed longer latency to leave the start box, and spent less time in the OF arena compared to those not fighting back, indicating anxiety-like behaviour. They also showed more fragmentation of sleep in SWS1 and SWS2. The results may suggest that rapid submission during SD may be more adaptive than surrender after a longer fight, given these outcome measures.

In conclusion, the studies presented in this thesis show that exposure to SD induced both short-term and long-term consequences for multiple behavioural features and at least short-term consequences for sleep. The behavioural consequences of SD are different from those of IFS. The studies generally support a high degree of face value for the SD model as a model for affective disorders, more relevant to anxiety than to depression.

List of publications

This thesis is based on the following papers.

Paper I. Kinn AM, Grønli J, Fiske E, Kuipers S, Ursin R, Murison R, Portas CM. 2008. A double exposure to social defeat induces sub-chronic effects on sleep and open field behaviour in rats. Physiology & Behavior, 95(4), 553-561.

Paper II. Kinn Rød AM, Milde AM, Grønli J, Jellestad FK, Sundberg H, Murison R. 2012. Long-term effects of footshock and social defeat on anxiety-like behaviours in rats: Relationships to pre-stressor plasma corticosterone concentration. Stress 15(6), 658-670.

Paper III. Kinn Rød AM, Murison R, Mrdalj J, Milde AM, Jellestad FK, Øvernes LA, Grønli J. Effects of social defeat on sleep and behaviour: importance of the confrontational behaviour. Physiology & Behavior
(resubmitted manuscript, PHB-D-13-00281R1)

Contents

Chapter 1

Introduction

1.1 General introduction

Social stressors are one of the main sources of stress in human life, especially for those low in the social hierarchy (Wood AM et al. 2012), and play a major role in the pathogenesis of affective disorders i.e. anxiety and depression (Taylor et al. 2011). Social stress can occur throughout the lifespan, from childhood neglect, abuse, and school bullying to work harassment in adulthood (Bjorkqvist 2001; Heim and Nemeroff 2001), or may be associated with traumatic events like violence and assault (Krug et al. 2002).

The interest for animal models of social stress has recently increased, especially the social defeat (SD) model, possibly due to the recognition that social stress is highly associated with pathology and the acknowledgement that natural stress models have important translational value (Chaouloff 2013). The SD model is based on the natural conflict occurring when a male intruder rat (or mouse) eventually subordinates itself to an unfamiliar territorial resident conspecific. The effect of defeat is studied in the intruder rat, and may induce short-lasting and long-lasting alterations in behaviour and physiology.

1.2 The concept of stress

Living organisms have a complex set of mechanisms to maintain constancy of their internal environment and to preserve life. Our understanding of these

mechanisms started with Claude Bernard's concept of an internal environment, *milieu interne* (Bernard 1885). This was further elaborated by Walter Cannon (Cannon 1932) who coined the term *homeostasis*, meaning steady state. Homeostasis is the maintenance of a relatively constant internal environment by an array of mechanisms in the body. Cannon recognized that emotional as well as physiological disturbances activate a sympathoadrenomedullary response, the 'fight or flight' response, preparing the body for action. The concept of *stress* was first used in the biomedical literature by Hans Selye in the 1930s. Selye outlined the 'general adaptation syndrome', the consistent sequence of three stages of physical responses triggered by a stressor (Selye 1936).

Stress has been defined as a state in which homeostasis is threatened or perceived to be threatened (Chrousos and Gold 1992). Stressors are the physical or psychological stimuli which threaten homeostasis. Later, the term *allostasis* has been used in the literature, defined as the processes actively maintaining homeostasis (McEwen 2000, 2010). The hypothalamo-pituitary-adrenocortical (HPA)-axis and the sympathoadrenomedullary system are such adaptive processes and promote adaptation and coping. When the body is forced to adapt to adverse situations, the cost to the body is allostatic load (McEwen 2000, 2010). Frequent stress, failure to habituate to repeated challenges, inability to shut off responses and inadequate responses are conditions which may lead to allostatic overload, which consequently may cause pathology.

The definition of stress has been debated because nearly all activities of an organism directly or indirectly threaten homeostasis, and the stress response is also activated during rewarding behaviour like sexual behaviour and winning a social interaction (Buwalda et al. 2012). In a recent review of the stress concept, Koolhaas and colleagues (2011) emphasized that:

> *The use of the terms 'stress' and 'stressor' should be restricted to conditions and stimuli where predictability and controllability are at stake; unpredictability being characterized by the absence of an anticipatory response and*

loss of control being reflected by a delayed recovery of the response and the presence of a typical neuroendocrine profile. (p. 1292).

This definition of stress and stressors exclude the short lasting adaptive activation of the stress response (Koolhaas et al. 2011).

Stress is thought to be maladaptive and potentially pathogenic if the response is sustained and not adequately terminated (Ursin H and Eriksen 2010). It has been emphasized that stress should be considered as a process that includes the stimulus, the perceptual processing of the input and the behavioural and physiological output (response) (Levine 2005). This approach forms the basis of the cognitive activation theory of stress (CATS: Ursin H and Eriksen 2010).

Stress is hypothesized to induce a cascade of behavioural and neurobiological processes, with possible different time-courses for each process (Koolhaas et al. 1997b). Some of these processes return to baseline after a few hours, others take days or weeks, and possibly some of the processes are indefinitely changed, never to return to baseline (Koolhaas et al. 1997b). The different temporal dynamics of the various stress parameters imply that the physiological and behavioural state of the individual at one time-point after stress is different from its state at a later time-point. Additionally, the vulnerability to subsequent stressors may vary with the state of the individual at different points in time. Thus, the symptomatology will be different depending on the time of measurement after stress and on subsequent stressors.

1.3 The stress response system

The main components of the stress response system are the central nervous system, the peripheral nervous system consisting of the autonomic and the somatic nervous system, and the HPA-axis (for reviews see e.g. Chrousos 2009; Chrousos and Gold 1992; Vermetten and Bremner 2002).

When a threat or aversive event is registered by the central nervous system, either as an environmental stimulus or as a memory of the previous aversive experience, the immediate response involves increased sympathetic activation via the

autonomic nervous system (within seconds, Eriksen et al. 1999), which again leads to responses that are essential to prepare the body for fight or flight. Peripheral sympathetic activation increases heart rate, blood pressure, respiration and metabolism. Noradrenergic activation throughout the brain leads to enhanced arousal, vigilance, focused attention and increased activity of the HPA-axis. Additionally, activation of the amygdala by noradrenaline is important for memory-retrieval and emotional analysis of the stressor (McGaugh 2000). If the stressor is of a threatening nature, the amygdala activates the stress system (LeDoux 1994). Also, sympathetic activation of the adrenal medulla leads to release of adrenaline and noradrenaline, which have partly the same effects in the body as sympathetic activation. The parasympathetic arm of the autonomic nervous system counteracts the sympathetic arm to prevent an exaggerated response. The two arms of the autonomic nervous system are at all times activated, but the balance of activation leads to specific responses, like fight/flight, sleep/wakefulness, digestion, reproduction, and so forth (Chrousos and Gold 1992; McCarley 2004; Van Reeth et al. 2000; Vermetten and Bremner 2002).

The other and slower part of the stress response system is the HPA-axis. Neuroendocrine cells in the hypothalamus release corticotropin-releasing hormone (CRH) into the blood vessels surrounding the pituitary stalk, leading to the release of adrenocorticotropic hormone (ACTH) from the anterior lobe of the pituitary into the blood (within seconds, Eriksen et al. 1999). Cells in the adrenal cortex are stimulated by ACTH to release glucocorticoids into the bloodstream (within minutes, Eriksen et al. 1999). The main glucocorticoid is cortisol in humans and corticosterone in rats. Glucocorticoids have a negative feedback effect on the HPA-axis by down-regulating the release of CRH and ACTH, both directly and indirectly via the hippocampus. In addition to the increased release of glucocorticoids as a response to stress, glucocorticoids (like essentially all hormones) are released in a circadian pattern in both rats (Allen-Rowlands et al. 1980) and humans (Weitzman et al. 1971).

The complexity of the glucocorticoids is illustrated by its effect on behaviour, arousal, sleep, brain development and function, and bodily functions like the immune

system, endocrine systems and energy mobilisation, (Chrousos and Gold 1992; Lupien et al. 2009; Van Reeth et al. 2000).

1.4 The basics of sleep and sleep regulation

Exposure to stress may lead to altered behaviour and sleep. This is seen in human affective disorders that are associated with stress, and will be introduced below (1.5 Affective disorders associated with stress). Because the consequences on sleep are an important part of the thesis, the basics of sleep and sleep regulation will be described in some detail in the following.

Normal sleep in humans and animals comprises a complex combination of physiological and behavioural processes. Sleep may be defined as a reversible behavioural state of partial perceptual disengagement from and unresponsiveness to the environment (Carskadon and Dement 2011). However, the sleeping brain can be easily woken when given a sufficient level of stimulation, and to some degree it distinguishes important information from unimportant information, a crucial feature which makes it possible to wake up when danger is present (Portas et al. 2000).

Sleep and wakefulness in both humans and animals are objectively measured by polysomnography, a set of electrophysiological parameters where the core measures are the electroencephalogram (EEG – brain activity), electromyogram (EMG – skeletal muscle activity) and electrooculogram (EOG – eye movements, not often used in rats). On the basis of these parameters, the different sleep stages can be defined and quantified by a set of scoring criteria in humans (Rechtschaffen and Kales 1968) and rats (Neckelmann and Ursin 1993; Ursin R and Larsen 1983).

Normal sleep is divided into two main phases in all mammals: non rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Through the inactive phase, sleep progresses in cyclic patterns. One of these is the alternation between NREM and REM sleep, which has 4-5 cycles throughout the night in humans. Sleep starts in NREM and progresses through the sleep stages of NREM before entering the first REM sleep episode after about 90 minutes in humans (Carskadon and Dement 2011), and 12 minutes in rats (McCarley 2007). Characteristic for the NREM sleep in both humans and rodents is reduced EMG

activity and the presence of sleep spindles and high-voltage slow waves (delta waves) in the EEG. In humans, NREM is classically subdivided into stages 1-4 (Rechtschaffen and Kales 1968). Stages 3 and 4 contain the highest intensity of slow waves (high total power, amplitude, and incidence of slow waves), and is collectively named deep slow wave sleep (SWS). In rats, NREM sleep is subdivided into SWS1 and SWS2 (Ursin R and Larsen 1983), where SWS2 contains the highest amount of slow waves, and is comparable to deep SWS in humans. REM sleep is characterized by the occurrence of rapid eye movements shown in the EOG, desynchronized EEG similar to wakefulness, and very low EMG activity (atonia) with occasional muscle twitches. In rats, low EMG activity and the presence of theta activity obtained by intracranial EEG gives adequate characteristics for the REM sleep. Wakefulness is characterized by desynchronized EEG and high EMG activity. Visual scoring of the sleep recording provides detailed information of sleep pattern, for example sleep latency, total sleep time, time spent in each sleep stage, number of stage shifts (fragmentation) and number of awakenings. These parameters represent a good measure of sleep quality and quantity.

Information of EEG power during sleep may be obtained from power spectral analyses (e.g. fast Fourier transform), which describe power or energy distribution in each EEG frequency band. Power values reflect both the incidence and amplitude of waves. Slow wave activity (SWA) is the EEG power in the low-frequency/delta band (e.g. 0.5-4.5 Hz, a definition which may vary between studies in both humans and rats). Compared with wakefulness, the overall power of the EEG increases during sleep, reflecting greater synchrony of CNS activity, and is greatest during deep SWS (Greene and Frank 2010).

The timing and quality of sleep is regulated by the interaction between sleep need (homeostatic factor), circadian factors and behaviour (Ursin R 2008). The homeostatic factor accumulates during time spent awake (Borbely 1982), and is reflected in the amount and intensity of the deep SWS (Achermann and Borbely 2003). The circadian factor (mediated by the suprachiasmatic nuclei) promotes sleep during certain periods of the day, and determines to a large degree the timing and duration of the sleep period (Dijk and von Schantz 2005). In addition to the

homeostatic factor and the circadian factor, deactivation by behaviour is necessary. Lying down in a safe environment facilitates muscle relaxation and reduced activation of the brain. Exposure to a stressful stimuli leads to activation of the stress system and stimulates arousal, which again supresses and alters sleep (Chrousos 2009; Van Reeth et al. 2000). Stress is thus a state where sleep homeostasis and sleep behaviour are threatened, and may induce changes in sleep regulation.

1.5 Affective disorders associated with stress

Although stress is naturally occurring and induces an adaptive response, it is also believed to play a major role in the pathogenesis of affective disorders. While most, if not all, people experience severe stress in the course of their lives, only a minority will develop a disorder (Kessler et al. 1995), apparently reflecting an abnormal response to stress rather than the norm. The reasons why some develop disorders, while others do not, may be explained by the diathesis stress hypothesis, which proposes that the interaction of diathesis (predisposition or vulnerability) to affective disorders and the experience of stressful events may result in psychopathology. The predisposition or vulnerability can involve e.g. a particular genetic makeup, physiology or personality, or a combination of these. In addition to these, the characteristics of the stressor or trauma play a role in development of a disorder or resistance/resilience to it (Monroe and Simons 1991). Anxiety and depression are complex affective disorders associated with stress.

Criteria for the classification of affective disorders and other mental disorders have been developed to provide guidance to clinicians and researchers, for example The Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV, American Psychiatric Association 1994), 4th edition-Text Revision (DSM-IV-TR, APA 2000), 5th edition (DSM-5, APA 2013), and the International statistical classification of diseases and related health problems (ICD-10, World Health Organization 2008).

1.5.1 Anxiety disorders

According to DSM-IV-TR (APA 2000) anxiety disorders are classified as phobias, panic disorder, obsessive-compulsive-disorder (OCD), generalized anxiety

disorder (GAD), acute stress disorder (ASD) and post-traumatic stress disorder (PTSD). Common for these is autonomic activation which induces symptoms like increased heart rate, high blood pressure, respiratory changes, altered metabolism, enhanced arousal, vigilance and focused attention. In the USA, the prevalence for anxiety disorders is reported to be for lifetime 28.8% and for 12-months 18.1% (Kessler et al. 2005a; Kessler et al. 2005b). Animal models of anxiety normally aim to mimic symptoms of GAD, ASD and PTSD.

Fear versus anxiety

Fear and anxiety are emotional states that mediate survival responses to threats (Porges 1995). In neuroscience, fear is commonly defined as an aversive reaction elicited by the perception of a *specific* threat stimulus, whether conditioned or unconditioned. Anxiety, in contrast, is commonly defined as prolonged hyper-vigilance in anticipation of, or response to, a diffuse or imagined threat where danger is not clearly imminent or not present (Sylvers et al. 2011). The states are similar as autonomic arousal occurs in both, but the relationship to HPA-activation is less clear for anxiety than it is for depression. There are also other important distinctions between fear and anxiety. The fear response dissipates quickly, whereas anxiety promotes a sustained response. The two responses are mediated by different brain regions: the central amygdala is the primary brain structure in fear, whereas the bed nucleus of stria terminalis is the primary brain structure in anxiety (Sylvers et al. 2011). Anxiety disorders as used in the diagnostic classification may thus be seen to include disorders of fear (e.g. phobias, social anxiety) *and* of anxiety (GAD, ASR, PTSD).

1.5.2 Depressive disorders

Mood disorders include conditions such as major depressive disorder (MDD), bipolar disorder, and dysthymic disorder (chronic mild depression) (DSM-IV-TR, APA 2000). Common for the mood disorders are disturbed mood as the predominant feature, and symptoms of altered behaviour and sleep. The prevalence of mood disorders is reported to be for lifetime 20.8% and for 12-months 9.5% (Kessler et al. 2005a; Kessler et al. 2005b). The most debilitating form of depression is MDD, and it

is the symptoms of MDD that most animal models of depression normally aim to mimic.

1.5.3 Altered behaviour associated with affective disorders

Corticotropin-releasing hormone has been hypothesized to have direct behavioural effects in the brain that lead to increased arousal, alertness, attention, and readiness (Vermetten and Bremner 2002). Abnormal levels of arousal are seen in both anxiety and depression. Depressed patients may show either hypo- or hyperarousal in the central nervous system (Nofzinger et al. 2000), while anxiety patients show hyperarousal (DSM-IV-TR, APA 2000). The clinical links to abnormal arousal include hypersomnia or insomnia (see 1.5.4 Sleep alterations associated with affective disorders below), increased or decreased psychomotor activity and increased startle response (DSM-IV-TR, APA 2000; Nofzinger et al. 2000).

Increased startle response is one of the diagnostic symptoms linked to increased arousal in ASD and PTSD (DSM-IV-TR, APA 2000). In GAD patients, negative emotionality is associated with an excessive startle response (Ray WJ et al. 2009). In PTSD patients, a lack of habituation of the startle response magnitude and skin conductance has been reported (Jovanovic et al. 2009; Metzger et al. 1999).

The temperament and character inventory (TCI) (Cloninger et al. 1993) and the earlier tridimensional personality questionnaire (Cloninger 1987) are questionnaires developed to evaluate the psychological and biological tendencies of human behaviour, and are widely used in psychiatry and psychology. Harm avoidance is one of the dimensions of temperament, an inherited personal trait which is stable over time. When exposed to potential threat, people with high scores on harm avoidance show caution and careful planning. Harm avoidance has been shown to positively correlate with symptoms of both anxiety and depression (Jylha and Isometsa 2006), and to correlate negatively with resilience (Kim et al. 2013). Patients with unipolar depression and PTSD show high harm avoidance (Jakšić et al. 2012; Young et al. 1995).

One of the core symptoms of MDD is anhedonia, defined as inability to find pleasure in things usually found enjoyable (DSM-IV-TR, APA 2000), e.g. recreational activities, eating, social interaction and sexual activities. Anxiety and

other neuropsychiatric disorders have also been associated with anhedonia (Der-Avakian and Markou 2012; Grillo 2012). Decreased sexual desire and increased sexual dysfunction are common symptoms with depression and anxiety disorders (Kendurkar and Kaur 2008; Kotler et al. 2000; Laurent and Simons 2009; Michael and O'Keane 2000), but may also be a result of the treatment (Fossey and Hamner 1994; Williams and Reynolds 2006).

1.5.4 Sleep alterations associated with affective disorders

Sleep alterations are among the symptoms for both anxiety and depression (DSM-IV-TR, APA 2000). In patients with GAD, ASD and PTSD, these subjectively reported sleep alterations are difficulty with initiation and maintenance of sleep (insomnia), which may be linked to increased arousal. Another common symptom in PTSD is the occurrence of distressing dreams about the triggering traumatic event. For MDD the reported symptoms are insomnia, hypersomnia (daytime sleepiness) or decreased need for sleep. In addition to these subjective alterations, objective alterations have been reported in several affective disorders.

There are few studies on objective sleep alterations in GAD patients (Monti and Monti 2000). Studies have reported increased sleep onset latency, increased wake time after initial sleep onset, lower sleep efficiency, and reduced total sleep time relative to controls. Findings of abnormalities in the amount and timing of REM sleep and the amount of SWS are inconsistent in GAD patients (Monti and Monti 2000; Papadimitriou and Linkowski 2005).

Objective findings on sleep disturbances in PTSD are also inconsistent, even the subjective reports of trouble initiating and maintaining sleep are inconsistently found in objective assessment of sleep. Reports of frequent nightmares in PTSD (which most typically arise during REM sleep) have focused interest on REM sleep alterations. Abnormalities in the timing or amount of REM sleep in PTSD have not been consistently found. However, increased REM density (frequency of rapid eye movements) and REM sleep fragmentation (arousals and stage shifts) have been reported (Kobayashi et al. 2007; Ramsawh et al. 2011).

Regarding objective sleep alterations, MDD is the most studied affective disorder. These include increased sleep onset latency, reduced total sleep time, lower

sleep efficiency, reduced REM sleep latency, increased amount of REM sleep, increased REM density, reduced amount of sleep stage 3 and 4 (deep SWS) and increased sleep fragmentation (Peterson and Benca 2011).

Changes in EEG power during sleep have also been reported in both depression and anxiety disorders, including reduced power in the low-frequency delta band (0.2-4 Hz) and increased high frequency power (>20 Hz) (Armitage 2007; Borbely et al. 1984; Tekell et al. 2005; Woodward et al. 2000).

1.5.5 Abnormalities in the stress response associated with affective disorders

Depression in humans is often associated with higher than normal basal levels of glucocorticoids, hypercortisolism (de Kloet et al. 2005; de Villiers et al. 1987). One possible mechanism underlying hypercortisolism is a reduced inhibition of the HPA-axis by the hippocampus. Decreased numbers of mineralocorticoid receptors and glucocorticoid receptors in the hippocampus, as seen for instance in adolescent and adult rats exposed to prenatal stress, weaken the inhibition of the stress response, resulting in increased basal and/or stress-induced glucocorticoid secretion (Lupien et al. 2009).

Post-traumatic stress disorder (PTSD) is often associated with low basal levels of glucocorticoids, hypocortisolism (Heim et al. 2000; Mason et al. 1986). However, hypocortisolism in PTSD is not a consistent finding (Eckart et al. 2009; Inslicht et al. 2006), although a meta-analysis did yield some evidence for hypocortisolism in a subgroup of people who seem to be at the greatest risk of developing PTSD (Meewisse et al. 2007). One proposed mechanism for the development of hypocortisolism in PTSD is increased CRH release, leading to a lower ACTH response to CRH, and resultant low levels of peripheral cortisol (Vermetten and Bremner 2002). The hypocortisolism in PTSD may be a pre-traumatic risk or vulnerability factor that is induced by genetic predisposition and/or early exposure to stress rather than a consequence of trauma (Pitman 1997), as suggested by the diathesis stress hypothesis.

1.5.6 Comorbidity

Among patients who meet criteria for major depression, 51% are also suffering from an anxiety disorder (Kessler et al. 1996). As described there is an overlap of diagnostic criteria and symptoms of anxiety and depression. Consequently, it is difficult to separate the disorders in humans, and thus also in animal models. There are a few possible explanations for this frequent comorbidity. One is that there may be a common underlying genetic and/or environmental factor predisposing to both conditions, which may manifest itself as anxiety or depression or both at different times in life. For instance, compared to individuals with two copies of the long allele, individuals with one or two copies of the short allele of the serotonin transporter promoter polymorphism exhibit more depressive symptoms, diagnosable depression and suicidality after exposure to stressful life events (Caspi et al. 2003), *and* have a higher risk of developing PTSD after adult traumatic events and childhood adversity (Xie P et al. 2009). Another explanation is that anxiety disorders may cause or contribute to the development of depression. This explanation is supported by reports that the age of onset for anxiety disorders is lower than for depression (Schatzberg et al. 1998). Additionally, life time MDD has been shown to be secondary to other mental disorders, whereas anxiety is the most common pre-existing disorder (Kessler et al. 1996).

1.6 Animal models of affective disorders

Today there is an increasing focus on affective disorders, how they impair the quality of life for the patients and how they impact on societal economies. Laboratory animal models can contribute to the understanding of the triggering environmental factors as well as the mechanisms, neurobiology and genetics behind the disorders. These models and knowledge from them can be used to improve prediction and treatment of the disorders.

Animal models are based on the foundation that all vertebrate animals, especially mammals, have through evolution developed substantial commonalities of structure and function, from gross anatomy to organ systems and the most elemental processes between and within cells. These commonalities imply that the brain and its

regulation of behaviour in any mammal will probably have substantial generalizability to all mammals, including humans (Overmier and Carroll 2001).

Criteria have been established for the evaluation of animal models of affective disorders. Widely quoted are the criteria for models of depression developed by McKinney and Bunney (1969), which are also utilized in models of other affective disorders. They proposed that the minimum requirements for an animal model of an affective disorder are: 1) It is 'reasonably analogous' to the human disorder in its manifestations or symptomatology; 2) there is a behavioural change that can be monitored objectively; 3) the behavioural changes observed should be reversed by the same treatment modalities that are effective in humans; 4) it should be reproducible between investigators.

McKinney and Bunney's criteria have later been further elaborated to account for etiological, face, predictive and construct validity (Henn and Vollmayr 2005; Willner 1984). An animal model should have similar causative conditions to the human disorder, *etiological validity;* similar manifestations and symptom profiles to the disorder state, *face validity;* similar treatment responses to that seen in the human disorder, *predictive validity*; and similar underlying neurochemical processes responsible for the symptoms observed in the human disorder, *construct validity*.

Diagnostic criteria for affective disorders like PTSD and MDD include symptom persistence over time, such as several weeks (DSM-IV-TR, APA 2000). Furthermore, symptoms may take time to manifest themselves following a precipitating event, e.g. delayed onset of PTSD. Thus it has been proposed that animal models for these disorders should show long-lasting changes (Stam 2007; Yehuda and Antelman 1993).

Severe stress is a common risk factor for affective disorders like PTSD and depression (Neria and Bromet 2000). Several animal models of affective disorders are therefore based on various forms of stress (etiological validity) with the aim to induce alterations of behaviour and physiology analogous to the human disorder (face validity). Some animal models are proposed to be specific for anxiety or depression. Others may model both anxiety and depression, which is not surprising as many symptoms of anxiety and depression overlap. Several animal models based on

environmental stress have been developed (see 1.6.2 Stress exposure as animal models of affective disorders below), and for each model a variety of protocols are used. One must have this in mind when studies are compared and lines are drawn to the human condition.

Patients with a given affective disorder have a set of behavioural and physiological symptoms characteristic for the disorder. They are diagnosed from these symptoms in addition to subjective verbally expressed symptoms. An animal model of the affective disorder aims to reproduce parts or all of the objective behavioural and physiological changes present in patients with the disorder, but cannot access subjective symptoms.

1.6.1 How to measure the face validity of animal models?

Several methods may be used to evaluate behavioural and physiological effects of animal models associated with anxiety and depression in humans, i.e. the face validity.

Over the years, a large number of tests of animal behaviour have been developed and validated as tests for anxiety-like or depression-like behaviour (Lister 1990; Overstreet 2012; Ramos and Mormède 1998). A test is said to be valid as a test for anxiety-like or depression-like behaviour if the effect is reduced by anxiolytic or antidepressant drugs, respectively. In the following there will be given an introduction to the behavioural tests used in this project.

To study the effect of sleep in rodents, measures of brain and muscle activity (EEG and EMG) are used. Sleep registration in rodents will be introduced after the introduction of behavioural tests.

Open field (OF)

The OF test consists of the measurement of behaviours elicited by placing the animal in a novel open space where escape is prevented by surrounding walls. Several variations of the apparatus and the protocol have emerged. The two main paradigms of the test are the forced exploration OF test, where the animal is placed directly in the arena, and the free exploration OF test, sometimes named the OF

emergence test, where the animal is allowed to explore freely from a start box or home cage.

A novel arena of any sort is likely to evoke complex, competing behavioural tendencies reflecting anxiety and fear (harm avoidance) on the one hand and exploration and curiosity (novelty seeking) on the other (Ray J and Hansen 2004). The original view was that a novel, potentially dangerous environment initiates a stress response leading to low locomotor activity and high defecation rate (Archer 1973; Denenberg 1969), an indication of increased sympathetic activity (Sapolsky 1998). Another measure used is activity or time spent in the central area. In a novel OF, the rats tend to move mostly in the peripheral area, where they can touch the walls, thereby avoiding the open, more aversive and potentially dangerous central area. An additional parameter used in the free exploration OF is latency to leave the start box/home cage, where long latency may reflect high anxiety.

Overall, the first exposure to the OF is more anxiety-provoking because of the novelty of the arena. Over repeated exposures, the field loses its novelty and habituation normally occurs, indicated by e.g. increased locomotor activity and decreased defecation (Archer 1973; Denenberg 1969). A lack of habituation in the OF may indicate a sustained state of anxiety.

Open field behaviour seen in rodents after exposure to stress parallels in many ways behaviours seen in humans with anxiety and/or depression. Avoiding the central area or high latency to leave the start box/home cage may be linked to the concept of harm avoidance (Jylha and Isometsa 2006; Ray J and Hansen 2004; Vermetten and Bremner 2002). Reduced total locomotor activity can however be interpreted as freezing behaviour reflecting fear/anxiety, or as psychomotor retardation reflecting a symptom in human depression. Increased locomotor activity may reflect the depression-like symptom of psychomotor agitation. Thus the OF test may test both anxiety-like and depression-like behaviour.

Stress-induced alterations in behaviour in the OF are reduced by some but not all anxiety reducing drugs (Prut and Belzung 2003). Additionally, behavioural alterations in the OF test following a stressor have been shown to be reversed by antidepressant drugs and by sleep deprivation (Katz et al. 1981; Meerlo et al. 1996a),

which has an acute antidepressant effect in humans (Wu and Bunney 1990). These effects on OF behaviour also indicate that the OF test may test both anxiety-like and depression-like behaviour.

Elevated plus maze (EPM)

The basis for the development of the EPM test was that rats display higher avoidance and lower exploratory behaviour in open elevated alleys compared to closed alleys (Montgomery 1955). The apparatus described by Pellow and colleagues (1985) consisted of four elevated arms, arranged in a plus shaped cross with two open and two enclosed opposing arms, connected by a central platform giving free access to all four arms. The rat is placed on the central platform and is allowed to explore the maze for a fixed amount of time. The most used parameters are related to entry, activity and duration on open arms. As in the OF test, repeated exposure to the EPM arena is likely to induce habituation, and a lack of habituation may indicate a sustained state of anxiety. Compared to the OF, the EPM apparatus is more standardized.

The EPM test has been validated by Pellow et al (1985) who found that rats consistently avoided the open arms and preferred the closed arms. Open arm approach was increased by anxiolytics, decreased by anxiogenic substances, and was unaffected by antidepressants. Thus, avoidance and lower exploration of open arms in the EPM is taken as an indicator of anxiety. As for central activity in the OF test, decreased exploration of the open arms may be interpreted as harm avoidance seen in human anxiety (Jylha and Isometsa 2006; Ray J and Hansen 2004). Taken together, the EPM test is viewed as a test of anxiety-like behaviour.

Acoustic startle response (ASR)

Startle is regarded as a preparatory reflexive behaviour. The ASR is enhanced in threatening situations or following an aversive event. In response to a loud noise, both animals and humans show a startle reflex by blinking the eyes and contracting skeletal muscles. In the ASR test the animal is put in a pressure sensitive tube inside a sound attenuated chamber. A series of intensive, sudden acoustic stimuli is presented to the animal and the magnitude of the muscular outcome of the ASR is measured by changes in pressure to the floor in the tube.

The startle response has a short latency (e.g. 8 milliseconds measured by EMG in the hind leg) and is thought to be mediated by a relatively simple neural pathway. In rats, the most accepted primary acoustic startle reflex pathway involves three central synapses: a) auditory nerve fibres to cochlear root neurons, b) cochlear root neuron axons to cells in the nucleus reticularis pontis caudalis, and c) nucleus reticularis pontis caudalis axons to motor neurons in the facial motor nucleus (pinna reflex) or in the spinal cord (whole body startle) (Davis 2006; Koch 1999).

In humans, the startle response has greater magnitude during negative affective states (Lang et al. 1998), and exaggerated startle response is among the DSM-IV-TR (APA 2000) diagnostic criteria for PTSD and ASD. Animal studies have shown sensitization of the ASR after footshock (enhancement of the response) (Davis 1989; Milde et al. 2003).

The sucrose preference test

When given a choice, rats as well as humans normally prefer to drink sweetened liquids. Katz (1982) reported that sucrose and saccharine consumption were reduced by chronic severe stress, indicating anhedonia, a depression-like symptom in rats. To test this stress-induced anhedonia in rats there are now several versions of this test. Animals are normally adapted to the sweet solution before the test. In one version of the test, rats are given a bottle of sweet solution for a short period (e.g. one hour). In the sucrose preference test rats are given a free choice between water and sucrose over several hours (usually 24 hours). Both sucrose and saccharin, a non-caloric artificial sweetener, have been used to test anhedonia. The use of sucrose in the test for anhedonia is a debated topic because the underlying motivation for sucrose intake may be both for caloric intake and for hedonic reasons, mediated by separate circuits in the brain (Bear et al. 2001). However, sucrose and saccharine preference tests have to a large extent been used interchangeably.

Reduced intake of a sweet solution is proposed to reflect decreased motivation and anhedonia, which is a core symptoms of depression (DSM-IV-TR, APA 2000), and is also associated with anxiety (Der-Avakian and Markou 2012; Grillo 2012). The sucrose preference test is normally viewed as a test of depression-like behaviour, however the test cannot be ruled out as a test of anxiety-like behaviour.

Sexual behaviour

Stress leads to a decrease in reproductive hormones and altered neurotransmission underlying reproductive behaviour. Stress may also lead to anhedonia and decreased motivation which may reduce sexual behaviour. In the sexual behaviour test, a male rat is introduced to a female rat in oestrus for a pre-defined amount of time, and sexual behaviour is scored in the gender of interest. Prolonged latency and decreased number of mounts, intromission, and ejaculation are considered signs of sexual dysfunction and decreased sexual motivation in male rodents (Argiolas et al. 1988; Hawley et al. 2011).

Humans diagnosed with anxiety and/or depressive disorders may report a reduction in libido and are at greater risk of experiencing physiological impairment in sexual functioning (e.g. ejaculatory and erectile dysfunction) (Kendurkar and Kaur 2008; Kotler et al. 2000; Laurent and Simons 2009; Michael and O'Keane 2000). Reduced motivation for sexual activities is, like reduced sucrose preference, a sign of anhedonia (Gorwood 2008). Changes in sexual behaviour in rats may thus reflect both anxiety-like and depression-like behaviour and physiology.

Sleep registration in rodents

The objective sleep alterations seen in anxiety and depression may also be seen in rats after exposure to stress. Electrodes for EEG recording are surgically implanted on the skull of the rat, and EMG electrodes are implanted in the neck muscle. Data recordings are made by connecting the rat to a freely moving cable and the recording equipment or by wireless transmission of the data from a surgically implanted device (see 2.14.2 Sleep recording procedures). Sleep and wakefulness is scored manually according to a set of scoring criteria (e.g. Neckelmann and Ursin 1993; Ursin R and Larsen 1983), by automatic scoring algorithms, or by a combination of the manual and automatic method.

Some of the objective sleep alterations seen in patients with anxiety and depression have been shown in animals exposed to early life stress, CMS and learned helplessness (Adrien et al. 1991; Dugovic et al. 1999; Grønli et al. 2004; Mrdalj et al. 2013).

1.6.2 Stress exposure as animal models of affective disorders

Widely used stressors in animal models of affective disorders are uncontrollable and unpredictable electrical footshocks.

The learned helplessness model was originally introduced by Overmier and Seligman (1967), and was based on the observation that following a high number of repeated inescapable shocks (64 shocks in the original paper), animals (originally dogs) will not try to escape from a situation even if it is possible. Animals which had previously learned to escape shock did not develop learned helplessness when exposed to inescapable footshocks followed by escapable shock (Seligman and Maier 1967). This is thought to parallel the attitude in depressed humans, that behaviour does not influence what happens next (Miller and Seligman 1975).

The learned helplessness model is known to produce depression-like symptoms in rodents e.g. agitated motor behaviour, REM sleep alterations, reduced body weight, diminished sexual behaviour, reduced intake of sweet solution and elevated corticosterone and CRH levels (Nestler et al. 2002; Vollmayr and Henn 2003). Antidepressant drug treatment, electroconvulsive shocks and cognitive training reverse learned helplessness and the depression-like symptoms, while anxiolytics drugs do not (Nestler et al. 2002; Seligman and Maier 1967; Sherman et al. 1982). Thus, the learned helplessness model is primarily known as an animal model of depression. The model is also suggested as an animal model of PTSD (Foa et al. 1992; Krystal et al. 1989; LoLordo and Overmier 2011), although this is questioned (Yehuda and Antelman 1993).

Another model using footshock is the *brief inescapable footshock (IFS) model*. In this model animals are exposed to a relatively short-lasting session with a low number of IFSs. The model was first described by Levine and colleagues (1973). Murison and Overmier (1998) showed that there was a qualitative difference between 10 shocks and 100 shocks delivered to rats. Rats exposed to 10 shocks showed anxiety-like behaviour (immobility) in the sudden silence test, while 100 shocks had no effect. Overall, the model is known to produce anxiety-like symptoms in rats, as shocked rats show lower activity in an OF (Van Dijken et al. 1992c), less exploration of the open arms and lower activity in the EPM, reduced social behaviour in the

social interaction test (Louvart et al. 2005), higher ASR (Milde et al. 2003), more immobility in the sudden silence test (Murison and Overmier 1998; van Dijken et al. 1992a; Van Dijken et al. 1992b; Van Dijken et al. 1992c) and short-lasting lower preference for a sweet solution (van Dijken et al. 1992a). Long-lasting behavioural changes induced by brief IFS are sensitive to treatment with (putative) anxiolytic agents, whereas no beneficial effect of antidepressant drugs is reported (Van Dijken et al. 1992b). In a previous study in our laboratory the diathesis stress hypothesis connected to PTSD and the HPA-axis was investigated. As expected, only rats with lower levels of corticosterone *prior* to brief IFS showed higher ASR (Milde et al. 2003). These data indicate that the behavioural consequences of a stressor may be related to pre-stressor levels of HPA-activity. Thus it has been argued that the brief IFS model is an animal model of anxiety, and more precisely PTSD (Stam 2007).

The predator stress model utilizes a stressor that is more naturalistic than footshock. In this model, rats are exposed to predator odour or threatened by a predator like a cat (but not physically attacked). Rats exposed to predator stress show anxiety-like behaviour in the following days and weeks, for instance increased ASR, and reductions in sexual behaviour, social interaction, weight gain and open arm activity in the EPM (Blanchard et al. 2003; Stam 2007). Anxiolytic and potentially anxiolytic drugs have been shown to modulate the elicited changes (Blanchard et al. 2003). Thus the predator stress model is known as an animal model of anxiety.

Chronic stress. The first chronic stress model of depression was developed by Katz (1981), where rats were exposed to several relatively severe unpredictable stressors. The harsh stressors applied raised ethical issues, leading to the development of the *Chronic Mild Stress* (CMS) model by Willner and colleagues (1987). In the CMS model rodents are repeatedly exposed to a set of various mild stressors across several weeks, supposedly mimicking the mild stressors humans are exposed to in everyday life (daily hassles). Rats exposed to CMS develop depression-like symptoms, for example sleep alterations, decreased sexual behaviour, weight loss, altered locomotor activity in the OF, decreased exploration, increased immobility in the forced swim test and reduced preference for sweet solutions (Grønli et al. 2004; Grønli et al. 2005; Willner 2005; Yan et al. 2010). The effects can be reversed by

chronic treatment with antidepressant drugs and electroconvulsive shocks, while a number of anxiolytic drugs have no effect in the CMS model (Vollmayr and Henn 2003). Thus the CMS model is known as an animal model of depression.

Early life stress models include *prenatal stress* and *maternal separation*. In the *prenatal stress* model, the mother is for instance exposed to restraint stress, producing depression-like symptoms in the offspring. In the *maternal separation* model, rat pups are deprived of maternal care. The length and numbers of the separations have been shown to differently affect the rat pups. Brief maternal separation (e.g. 10 minutes per day) is shown to increase the resistance to stress in adulthood. Long-term maternal separation (e.g. 3 hours per day) may induce abnormal maternal behaviour (neglect) (Meaney et al. 1985), and may produce depression-like and anxiety-like symptoms in the pups that last into adulthood. Sleep alterations, elevated glucocorticoids response to stress, vulnerability to learned helplessness and ethanol self-administration, increased locomotion and decreased open arm activity in the EPM are seen after long-term maternal separation (Huot et al. 2001; Mrdalj et al. 2013; Nestler et al. 2002). Environmental enrichment and antidepressant drugs (Paroxetine, also used as anxiolytic) have been shown to reverse the depression-like and anxiety-like symptoms (Francis et al. 2002; Huot et al. 2001), indicating that early life stress is an animal model of both anxiety and depression.

The animal model of social defeat (SD) is also suggested as an animal model of both anxiety and depression, and is used in this thesis.

1.7 The animal model of social defeat

One of the main sources of stress in human life is of a social nature, like low ranking in the social hierarchy (Wood AM et al. 2012). Social defeat is associated with affective disorders i.e. anxiety and depression (Taylor et al. 2011). Studies in humans most often focus on school bullying and work harassment (Bjorkqvist 2001), and may be associated with traumatic events like violence and assault (Krug et al. 2002).

The animal model of social defeat (SD), most often using male rodents, is based on the resident-intruder paradigm first introduced by Ginsburg and Alle (1942).

A male rat intruder is placed in the territory of a bigger, older and more aggressive male resident rat. The intruder is attacked and defeated as indicated by fleeing, freezing and submissive behaviour (see Figure 1a,b). Behaviour and physiology are studied in the defeated intruder at different time intervals after stress exposure. Male rats are used in the social defeat model, as female rats do not normally show this aggressive territorial behaviour.

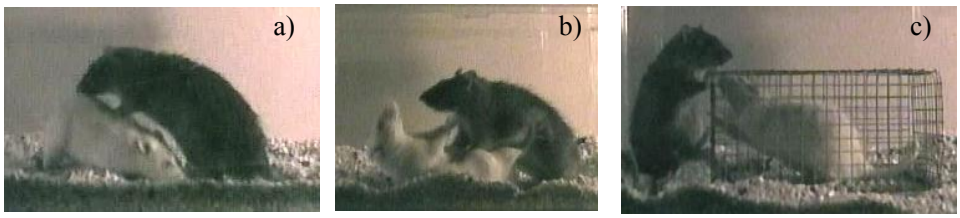

Figure 1. The white SD rat was **(a)** introduced, **(b)** defeated and further **(c)** exposed to the brown aggressive dominant rat. Total exposure to SD was 1 hour on one or two consecutive days.

Several variations of the SD model have been used. The nature of the social conflict may vary between only 'physical attack', and both 'physical attack' and 'threat of attack'. The 'physical attack' phase is when the intruder is exposed to the resident and attacked. After being defeated, the intruder may be physically separated from the resident, protected from repeated attacks and potential injuries, but still being under 'threat of attack' by having auditory, visual and olfactory contact with the resident (see Figure 1c). This time under threat of attack is known to be highly stressful (Tornatzky and Miczek 1994). The number of exposures to SD varies from a single exposure to daily exposures, lasting for minutes, hours or even for weeks. In the present project, the main stressor for the intruder was SD for a total of 1 hour, including 'physical attack', subordination and further exposure by 'threat of attack' protected by a wire mesh cage. The intruders were exposed to SD on one or two consecutive days, respectively single SD and double SD.

Single or double exposure to SD in rodents has been shown to induce acute, short-lasting and long-lasting changes on behavioural, physiological and neuroendocrine parameters. Some effects of SD may be evident both acutely and last for days and weeks, and some may not be present acutely, but develop over time. In

the present project the effects of single and double SD were examined on day 1 and up to day 24 after defeat.

1.7.1 Effects of single or double social defeat in rats

Acute effects of SD are shown during the social interaction and return to baseline during the hours after the termination of stress. These effects include increased corticosterone, ACTH, noradrenaline and adrenaline levels, increased core body temperature and increased heart rate (Heinrichs et al. 1994; Koolhaas et al. 1997b; Sgoifo et al. 1996; Tornatzky and Miczek 1994). Such acute effects were not studied in the present project.

In the OF test, rats exposed to single SD have decreased activity day 1, 2 and 7 after defeat (Meerlo et al. 1996a). In the same study a long-term effect appeared 28 days after defeat, as single SD rats showed increased latency for moving from the centre (where initially placed) to the periphery, compared to controls. Single SD has been shown to induce decreased central activity compared to controls when tested 7 days after defeat (Kavushansky et al. 2009). In a free exploration OF test, individually housed (compared to co-housed) single SD rats showed a longer latency to leave the home cage and less activity in the peripheral zone 21 days after defeat (Ruis et al. 1999). The latter effect was also seen on day 2 after defeat. However, effects of single SD on OF behaviour are not consistent, as some studies have shown a lack of short-term and/or long-term effects (Carnevali et al. 2012, (day 9 and day 21 after SD); Kavushansky et al. 2009, (day 1 after SD)). In the EPM test, studies have reported reduced percentage of time spent on open arms acutely after single SD (Heinrichs et al. 1992), an effect reversed by a CRH antagonist and by the anxiolytic midazolam. Another study showed increased latency to enter an open arm, reduced entries and time spent on open arms in the EPM 7 and 21 days after single SD (Carnevali et al. 2012). Reduced time spent on open arms has also been seen 14 days after single SD if the rats were housed individually and not in groups (Nakayasu and Ishii 2008; Ruis et al. 1999). However, there has also been reported unchanged activity on open arms or total activity in the EPM both 1 and 7 days after single SD (Kavushansky et al. 2009). Decreased preference for sucrose has been shown to develop day 22 after single SD (Carnevali et al. 2012), but also no preference for a

sweet solution have been reported (Meerlo et al. 1996b).The effect of single SD on ASR and sexual behaviour has not previously been reported. Sleep in SD rats has previously only been studied acutely after single SD. One study showed increased SWA during the active (dark) phase immediately following defeat, an effect that gradually vanished during the following inactive phase (Meerlo et al. 1997). Another study showed that SD rats kept awake by gentle handling after defeat, compared to controls kept awake by gentle handling during the same period, had a higher increase in SWA during NREM sleep (Meerlo et al. 2001). Responses of the HPA-axis i.e. corticosterone and ACTH are acutely increased after SD (Heinrichs et al. 1994; Koolhaas et al. 1997b; Sgoifo et al. 1996). Following single SD and a set of behavioural tests (OF, EPM and forced swim test), higher corticosterone levels have been seen compared to controls on day 7 after defeat, an effect that was not present day 1 (Kavushansky et al. 2009).

The predictive validity of single SD has previously been tested. Fluoxetine (antidepressant and anxiolytic drug) has shown to reverse body weight loss, reduced food intake and anxiety-like behaviour in the EPM (Berton et al. 1999). Clomipramine (antidepressant and anxiolytic drug) has reversed anxiety-like behaviour (immobility) in the sudden silence test (Koolhaas et al. 1990). Reduced locomotion in the OF has been reversed by sleep deprivation (Meerlo et al. 1996a). Anxiety-like behaviour in the EPM has been counteracted by a CRH antagonist and by the acutely anxiety reducing agent midazolam (Heinrichs et al. 1992).

To sum up, following single SD, rats in the OF normally show decreased total activity, decreased central activity and increased initial latency to move to a new sector. In the EPM they show reduced time on open arms and increased latency to enter an open arm. Single SD rats show reduced sucrose preference, and acutely increased SWA during sleep. These effects may be seen up to 4 weeks after SD, and may be associated with both anxiety-like and depression-like symptoms. Single SD may thus be regarded as an animal model of both anxiety and depression.

The effects of double SD have only been considered in a few studies. One study has shown that, compared to controls, both single and double SD induce reduced social interaction with a non-aggressive opponent, reduced total activity in

the OF, but no effect on central activity in the OF and no preference for sweet solutions (Meerlo et al. 1996b). The only difference between single and double SD was a more pronounced decrease in food intake and reduction in body-weight gain in the double SD rats. Like studies on single SD (Nakayasu and Ishii 2008; Ruis et al. 1999), reduced time spent on open arms was seen 14 days after double SD, if the rats were housed individually but not in groups (Nakayasu and Kato 2011). Regarding ASR, sexual behaviour and sleep, the effect of double SD has not previously been studied. The cited studies indicate that single and double SD induce qualitatively similar effects.

1.7.2 Effects of multiple exposures to social defeat in rats

Startle response and sexual behaviours have only previously been studied after multiple exposures to SDs (>2). Increased ASR was seen 10 days after a fourth exposure to SDs of 1 hour duration (Pulliam et al. 2010). A study using brief defeat with 5-10 minutes duration reported no difference in ASR after the fifth defeat (Miczek 1991). Reduction in sexual behaviour has been shown after several exposures to SD (Niikura et al. 2002), an alteration also seen in mice (Yoshimura and Kimura 1991).

Daily exposures for 12 days to several weeks, or continuous exposure for at least 10 days are regarded as chronic SD stress (Becker et al. 2008; Iio et al. 2012; Rygula et al. 2005). Chronic exposure induces decreased OF activity, increased immobility in the forced swim test, decreased home cage activity and decreased sucrose intake (Iio et al. 2012; Rygula et al. 2005), but failed to induce anxiety-like behaviour in the EPM (Rygula et al. 2008). Antidepressant drugs, but not an anxiolytic, have been shown to reverse altered behaviours after chronic SD (Rygula et al. 2006; Rygula et al. 2008). Thus, chronic SD is recognized as an animal model of depression.

1.7.3 Social defeat versus inescapable footshock

Social defeat is regarded as a ‘natural’ stressor, while IFS is criticized as being ‘unnatural’ or beyond the specie’s normal experience, reducing the etiological validity of the model (Koolhaas et al. 1997b). However, similar effects of single SD

and IFS have been seen. Both single SD and IFS induce lower activity in an OF (Meerlo et al. 1996a; Van Dijken et al. 1992c), less exploration of the open arms in the EPM (Carnevali et al. 2012; Louvart et al. 2005), reduced social behaviour in the social interaction test (Louvart et al. 2005; Meerlo et al. 1996b) and more immobility in the sudden silence test (Koolhaas et al. 1990; Murison and Overmier 1998; van Dijken et al. 1992a; Van Dijken et al. 1992b; Van Dijken et al. 1992c). Both stressors induced progressive and long-lasting effects on behaviour, although the effects were seen in different behavioural tests (Koolhaas et al. 1990; Van Dijken et al. 1992c). To evaluate if the two stressors differ, they need to be compared in the same study, with identical behavioural tests and time-points of testing.

1.7.4 Subgroups of social defeat rats show different vulnerability

When studying SD it may be important to include an evaluation of subgroups based on how the rats actually behave during the confrontation. Rats that show quick submission and passivity seem more affected by the defeat than those that fight back or oppose the resident during the social conflict (Meerlo et al. 1999; Stefanski 1998; Walker et al. 2009; Wood SK et al. 2010; Wood SK et al. 2013). Rats with this passive strategy during defeat display a higher corticosterone response to defeat and a higher level of neuronal activation in the amygdala and medial prefrontal cortex (Walker et al. 2009). They also show longer-lasting disturbances in diurnal heart rate, body temperature and locomotor activity rhythm, more body weight loss and different stress-induced immune changes than fighters (Meerlo et al. 1999; Stefanski 1998).

1.8 Aims of the study

The main purpose of the project was to study the face validity of the SD model for affective disorders by investigating short-term (Papers I, II and III) and long-term (Papers II and III) consequences for behaviour and sleep after single (Paper II) or double (Papers I and III) exposure to SD in rats. In particular the intention was to evaluate if SD can reproduce the alterations in locomotor activity, harm avoidance, startle response, anhedonia, sexual behaviour and sleep parallel to those observed in patients with affective disorders. Repeated measures of multiple behaviours and sleep were acquired in an attempt to distinguish between short-term and long-term effects.

Even if it is difficult to clearly distinguish anxiety from depression in humans due to the high comorbidity and the overlap of symptoms, it was an aim to determine if the face validity found in this study was overall directed towards an animal model of anxiety or depression or both.

Secondary aims of the project were:

1) to compare effects of SD to the effects of IFS (Paper II)

2) to explore the relationship between levels of corticosterone prior to SD or IFS stressor and the different post-stressor behaviours (Paper II)

3) to investigate differences in effects of SD on behaviour and sleep in two subgroups of rats with different coping styles in the SD (Paper III).

1.8.1 Hypotheses

The main hypothesis was that SD would induce short-term and long-term effects on behaviour and sleep parallel to those in human affective disorders. The behavioural effects were expected to include alterations of behaviour in the OF test (free and forced exploration), EPM test, ASR test, sucrose preference test, and sexual behaviour test.

More precisely it was expected that, compared to controls, SD rats would show lower central activity, lower total activity in the forced exploration OF test; longer latencies to leave the start box and less time in the open arena in the free exploration OF test (OF emergence test); lower activity on open arms and lower total activity in the EPM test; more defecation in the various OF tests and in the EPM test; higher ASRs; lower sucrose preference; and less sexual behaviour. A lower degree of habituation (response decrement) was expected. It was also hypothesized that SD would induce greater corticosterone responsiveness to the free exploration OF test.

Regarding sleep, the hypotheses were that SD rats would show alterations in sleep parameters such as increased sleep onset latency, reduced total sleep time, lower sleep efficiency, reduced REM sleep latency, increased amount of REM sleep, reduced amount of deep SWS (SWS2), increased sleep fragmentation (arousals and stage shifts), reduced power in the low frequency delta band (0.5-4.5 Hz) and increased high frequency power.

A secondary hypothesis was that SD and inescapable footshock (IFS) would have similar short-term and long-term effects on behaviour, and that these would be similarly related to pre-stressor levels of corticosterone, i.e. that rats with a low pre-stress corticosterone level would show the highest startle response as previously described for IFS rats by the research group (Milde et al. 2003).

Another secondary hypothesis was that rats showing quick submission during the SD confrontation would show the greatest alterations in behaviour, corticosterone responsiveness, sleep and EEG power.

Chapter 2

Methods

An overview of the methods used in the project is presented below. For more details, see each individual paper.

2.1 Ethical authorization

The experiments described in this thesis were approved by the Norwegian Animal Research Authority and registered by the authorities. The experiments have thus been conducted in accordance with Norwegian laws and regulations controlling experiments in live animals. Norway has signed and ratified The European Convention for the protection of Vertebrate Animals used for Experimental and other Scientific purposes, of March 18, 1986.

2.2 Design and procedures

2.2.1 Design Paper I

Rats in the social defeat (SD) group were exposed to double SD, one defeat on Day −1 and one on Day 0. Meanwhile, rats in the control group were left undisturbed. Effects on sleep and sexual behaviour were assessed in separate experiments to avoid changes in sleep due to sexual behaviour (Vazquez-Palacios et al. 2002).

In *Experiment 1* the effects of SD on sleep and OF behaviour were studied. Sleep recordings were carried out in all rats on three days: Day -2 for baseline, Day 1,

(13 hours after SD) and Day 4. A forced exploration OF test was performed on four consecutive days: Day 8, Day 9, Day 10 and Day 11.

In *Experiment 2* the effects of SD on sexual behaviour were studied. Sexual behaviours were tested on Day 1 and Day 4 after SD.

The SD procedure and behavioural testing in the OF, and the sexual behaviour test took place during the active (dark) phase, while sleep recording took place during the inactive (light) phase.

2.2.2 Design Paper II

Blood sampling for pre-stress corticosterone and adaptation to the sucrose preference test were carried out on Day -7 and Day -4 respectively, before the stress procedures were performed. Rats were exposed to single SD, inescapable footshock (IFS) or control procedures on Day 0. Sucrose preference tests started on Day 2 and were repeated on Day 9, Day 16 and Day 24. Body weight was assessed after each sucrose preference test. Rats were tested in the OF on Day 7, Day 14 and Day 21. They were tested in the EPM on Day 8, Day 15 and Day 22. The acoustic startle response (ASR) test was conducted on Day 19.

The SD procedure was conducted during the active phase, while the IFS procedure, control procedure and behavioural testing in the OF, EPM and ASR took place during the inactive phase. Sucrose preference started in the beginning of the active phase and lasted during the active and the inactive phase.

2.2.3 Design Paper III

Blood samples for corticosterone measures were taken before surgical procedures. Rats in the SD group were exposed to double SD, one defeat on Day −1 and one on Day 0. Meanwhile, rats in the control group were left undisturbed. Sleep recordings were carried out Day -1 (for baseline), Day 1, Day 7, Day 14 and Day 21. Rats were tested in the OF on Day 9, Day 16 and Day 23. A blood sample for corticosterone measures was drawn 5 minutes after the last OF test. ASR was tested on Day 24. The SD procedure was conducted during the active phase, while sleep recording and behavioural testing in the OF and ASR took place during the inactive phase.

	Paper I		Paper II		Paper III	
Day	Inactive phase	Active phase	Inactive phase	Active phase	Inactive phase	Active phase
Pre			Cort.		Cort.	
-2	Sleep rec.					
-1		**SD**			Sleep rec.	**SD**
0		**SD**	**IFS**	**SD**		**SD**
1	Sleep rec.	Sex. behav.			Sleep rec.	
2				SPT		
3			SPT& BW			
4	Sleep rec.	Sex. behav.				
5						
6						
7			OF		Sleep rec.	
8		OF	EPM			
9		OF		SPT	OF	
10		OF	SPT & BW			
11		OF				
12						
13						
14			OF		Sleep rec.	
15			EPM			
16				SPT	OF	
17			SPT & BW			
18						
19			ASR			
20						
21			OF		Sleep rec.	
22			EPM			
23					OF & Cort.	
24				SPT	ASR	
25			SPT & BW			

Table I: Experimental design. An overview of experimental design for the three experimental groups: social defeat, (SD), inescapable footshock (IFS) and control. Procedures in the inactive (light) phase and the active (dark) phase were identical on all days in all groups except on Day -1 and 0 when the different stress and control procedures were conducted.
ASR – acoustic startle response; Cort. – blood sampling for corticosterone measurement; BW – body weight; EPM – elevated plus maze; IFS – inescapable footshock; OF – open field; SD – social defeat; Sex. behav. – sexual behaviour; Sleep rec. – sleep recording; SPT – sucrose preference test.

2.3 Animals and housing

Experiments were performed on male Wistar rats (Taconic, Denmark). After arrival, rats were separated and housed individually and allowed acclimatisation before handling. For Paper I, cages were conventional Makrolon type III cages with a grid top. For Paper II and III rats were housed in individually ventilated cages (IVC), polypropylene Euro-standard Type III H. All rats were housed under a 12:12 hour light/dark schedule with progressive increase in light and dimming. The average ambient temperature was 22 °C and the relative humidity was 45-65%. The rats had free access to food and water. Bedding was changed once a week and never on the day before sleep recording or behavioural testing. For blood sampling, stress protocols and behavioural testing the rats were moved in their home cages to the dedicated room, and thereafter returned to the colony room.

The resident male rats (*BDIX*, for Papers I and III; Wistar rats, Paper II) used in the SD procedures were older and larger than the experimental rats. In order to stimulate territorial behaviour, they were each housed together with an ovariectomized female in polypropylene Euro-standard Type IV S cages for at least two weeks prior to the SD procedure. Resident rats had similar housing conditions to the experimental rats. In Paper III, females were brought into an oestrous cycle during the two weeks prior to SD by subcutaneous injections of oestradiol benzoate (200 µg/rat in oil) every fourth day and progesterone (0.5 mg/ rat in oil) 42 hours after the oestradiol injection. This was done to further increase the territorial behaviour of the male residents (Albert et al. 1988). The bedding was not renewed for at least 2 days prior to a social conflict in order to preserve the residents' scent.

2.4 Corticosterone measurements

Blood sampling for corticosterone measurements was performed in Papers II and III. Blood sampling took place between 09:00 and 12:00 hours as the natural circadian rhythm of corticosterone release in rats is low and stable during this period (Allen-Rowlands et al. 1980). Rats were placed in a sealed anaesthetic chamber and anaesthesia was induced with Isofluran. After visible muscle relaxation, the rat was placed on a table in a ventral position. One hind limb was shaved and smeared with

Vaseline before the saphenous vein was punctured and blood was collected in tubes. The whole sequence of moving the rat from the home cage to a complete blood collection took less than 3.5 minutes. The blood samples were left for ½ to 1 hour in room temperature for coagulation before they were centrifuged at 3500 rpm (1600 x g) for 10 minutes. Serum was separated and then frozen at -20^{o} C until analysis. The analysis was performed by means of Rat Corticosterone Enzyme Immunoassay. Serum samples were analysed in duplicate and measures were averaged.

2.5 Grouping

In Paper I, the rats were randomly divided into an SD group (n's=8 for both experiments) and a control group (n=8 and n=6 in experiment 1 and 2, respectively).

In Paper II, the rats were first divided on the basis of the pre-stress corticosterone measures: high corticosterone (239.32±15.82 ng/ml) and low corticosterone (32.60±5.07 ng/ml). Thereafter, rats from the two divisions were randomly distributed into an SD group, an IFS group and a control group, yielding 3 treatment groups (n's=20) and 6 subgroups (n's=10) where treatment groups were divided into high and low corticosterone.

In Paper III, rats were randomly divided into an SD group and a control group (n's =10). On the basis of the behaviour SD rats displayed during the SD confrontation, SD rats were split into two subgroups. SD rats showing *no* resistance during the two confrontations were assigned to the SD submissive (SDS, n=5) subgroup. SD rats fighting back during one or both of the SD confrontations, keeping the resident down in a supine posture, were assigned to the SD fighter (SDF, n=5) subgroup.

2.6 Social defeat (SD) procedure

The SD procedures were conducted under dimmed light (Paper I) or red light (Papers II and III). Female rats were removed from the residents' cage before SD confrontations. The residents had been trained to fight for their territory for at least 5 training sessions confronting younger males (Wistar), and were chosen for the SD based on short defeat latency.

SD procedures were adapted from the University of Groningen (Meerlo et al. 1996a), Haren, the Netherlands, after an internship with the group of professor Jaap Koolhaas. Rats from the SD group were individually placed in the cage of a resident rat. As soon as the SD rat showed a submissive posture (lying motionless on its back) it was placed in a small wire-mesh cage and re- introduced into the resident's cage to induce threat of attack and protection from repeated attacks and potential physical injuries. The total exposure time was one hour, including time in physical exposure and time placed in the protective wire-mesh cage. SD rats were exposed to an SD confrontation once (Paper II) or on two consecutive days (Papers I and III). Rats exposed to double SD were always confronted with two different residents to prevent possible adaptation effects.

In Paper III, behaviour of the intruder during the period of direct exposure to the resident was manually scored offline (Observer XT). The following measures were scored in the SD interaction, based on behavioural parameters from Koolhaas et al. (2013): Number of *received attacks*; *Submissive posture*; *Resident in supine posture*; *Initiated attack*, and duration (secs) of: *Received lateral threat*; *Flight*; *Freeze*; *General activity* (including received ano-genital sniffing, social explore, non-social explore, move away and rearing); *Hold resident down*; *Move towards resident*; *Upright posture*; *Total duration of direct exposure* (time from placing the intruder and the resident together, to time of separation with wire-mesh cage).

2.7 Inescapable footshock (IFS) procedure

In Paper II the IFS procedure was used as a stressor in the rats. The effect of IFS was compared to the effect of single SD on the various behaviours tested.

The shock apparatus consisted of a shock chamber containing a grid floor placed inside a sound attenuated cubicle. Footshocks were delivered through the floor by a computerized shock system with specialized software (Graphic State 3.0). Rats from the IFS group were exposed to 10 inescapable uncontrollable footshocks of 1mA intensity, each of 5 seconds duration. The inter-shock interval varied from 24 to 244 seconds (mean 90 seconds).

2.8 Control procedures

The control rats were left undisturbed (Papers I and III) or gently handled in the colony room for 1 minute (Paper II).

2.9 Open field (OF)

The OF test was conducted in Papers I, II and III with some differences.

For all experiments the OF arena was dimly lit and consisted of black walls surrounding a black square base ($1m^2$) divided into a peripheral sector (20 cm along the walls) and a central sector (a 60 cm^2 area in the middle). During the test, rats were left undisturbed in the room. After, rats were returned to their home cage and number of droppings recorded. The arena was thoroughly cleaned between each test with an ethanol solution (5% or 20%). Apart from this, the OF tests differed between the experiments.

In Paper I, the base of the open field apparatus was divided into 25 equally-sized squares by white stripes and organized into peripheral and central sectors. The rats were placed individually in the centre of the OF and activity was recorded for 6 minutes via a digital video camera for further analysis. OF activity was manually scored (Somnologica 2.0.2.). An activity score was considered when a rat crossed a sector border with both hind limbs. The following parameters were scored: *Peripheral sector activity;* C*entral sector activity; Total activity; First minute activity; Latency to enter the peripheral sector; Number of faecal droppings; Habituation of central activity in the OF (*expressed as percentage change from Day 8 to Day 11).

In Paper II, the forced exploration OF tests were conducted by individually placing the rats in the centre of the OF arena and recording their activity for 6 minutes by a digital camera. Activity was automatically analysed (EthoVision 3.1). The following parameters were analysed: *Total distance moved (*cm, expressed as total activity in the following*)* and *Number of faecal droppings*.

In Paper III, the free exploration OF tests (OF emergence tests) apparatus consisted of an OF arena with a small black start box located in the centre of the arena. The rats were placed individually in the start box, left undisturbed and activity was recorded for 15 minutes with a digital camera. With this method the rat was

allowed to *freely* explore the OF after leaving the start box. The position of the rat in the arena was manually scored (Observer XT). The following measures were assessed in the OF test: *Latency to exit the start box (sec); Percentage time spent in the arena (%); Number of faecal droppings* (in the start box and the arena summed).

2.10 Elevated plus maze (EPM)

The EPM test was used in Paper II and was conducted during the inactive phase.

The apparatus consisted of a plus shaped platform elevated above the floor. The four arms were connected by a central platform that gave access to the four arms. Two opposing arms were open and two were enclosed by black walls. Both open and closed arms were dimly lit. A rat was placed on the central platform, facing a closed arm, and was allowed to explore undisturbed for 6 minutes. After completion, the rat was returned to its home cage and faecal pellets were counted. Activity was recorded with a digital camera and automatically analysed (EthoVision 3.1). The following parameters were analysed: *Total distance moved in the EPM (*cm, expressed as total activity in the following*); Distance moved on open arm* (cm, expressed as activity on open arm in the following*); Percentage time spent on open arms (%,* time on open arm/ time on open + closed arm) × 100); *Latency to enter an open arm (sec)*: manually scored when all four paws were on the open arm; *Defecation in the EPM*.

2.11 Acoustic startle response (ASR)

The startle test was used in Papers II and III, and test procedures were adopted from Milde et al. (2003).

The startle apparatus consisted of a transparent cylinder placed on a pressure-sensitive plate that registered the rat's gross body movements. The cylinder was placed in a sound-attenuated chamber. All acoustic stimuli were presented through a speaker mounted above the cylinder. Stimulus delivery and recording were controlled by a computer using the SR-LAB software. Rats were placed in cylinders and chambers, and were left undisturbed for a 5 minutes habituation period with a background noise level of 67 dB. During the subsequent 10 minutes rats were exposed to identical series of 30 acoustic stimuli, 10 each of 95, 105 and 115 dB,

presented pseudo-randomly with variable inter-stimulus intervals. The ASR protocol was the same for all rats in both Paper II and Paper III. For each ASR, *Maximum response amplitude (Vmax)* was recorded.

2.12 Sucrose preference and body weight

A sucrose preference test was used in Paper II.

Prior to stress and control procedures rats were adapted to the sucrose preference test for 24 hours. During the sucrose preference tests, rats were offered a free choice for 24 hours (starting in the active phase) between two bottles, one containing a 1% sucrose solution and the other containing tap water. The consumption of sucrose solution and water was measured by weighing the bottles before and after the test. The dependent variable was *Sucrose preference*, defined as the amount of sucrose solution consumed as a percentage of the total fluid consumption. Rats were weighed after the sucrose adaptation and after each sucrose preference test.

2.13 Sexual behaviour

A sexual behaviour test was used in Paper I, and test procedures were adapted from Grønli et al. (2005).

To ensure receptivity to the male rat, the ovariectomized female rats (Brown Norway) were brought into oestrus before the sexual behaviour test and training. Pre-experimentally, each male rat underwent 3 sexual training sessions and was excluded from the experiment if ejaculation was not reached. During the 30 minute sexual behaviour test a female in oestrus was introduced into the home cage of the male rat, and male sexual behaviour was scored online (Somnologica 2.0.2). The following measures of sexual behaviour were scored: *Latency* and *Frequency* of *Mounting*, *Intromissions* and *Ejaculations*.

2.14 Procedures and analysis of sleep recording

Sleep was recorded in Papers I and III.

2.14.1 Surgical procedures

For Paper I, rats weighing 200g, were anesthetized by subcutaneous (sc) injection in the neck with a mixture of Hypnorm and Dormicum diluted with distilled water. They were implanted with stainless steel screw electrodes for EEG recording and silver wires in the neck muscle for EMG recording. All electrodes and the silver wires were connected to a socket.

For Paper III, all experimental rats were given antibiotic, 5 ml Bactrim per 250 ml drinking water for 3 preoperative days. Rats weighing 300g were anesthetized by sc injection in the neck with a mixture of Hypnorm and Midazolam, diluted with distilled water. They were implanted with a sc telemetry transmitter with biopotential leads for EEG and EMG recordings. Incisions were made in the dorsomedial lumbar region for the transmitter and on the skull for the biopotential leads of EEG and EMG. The leads for EMG recording were attached to the neck muscle. The incision in the dorsomedial lumbar region was closed with wound clips, and the skin on the head was closed with interrupted mattress sutures.

For both experiments, the EEG deviations were bilateral fronto-frontal (FF) and fronto-parietal (FP). The leads were placed epidurally in drill holes. EEG electrodes were secured to the skull with dental acrylic.

After surgery, rats received one analgesic sc dose of Temgesic, followed by analgesic sc doses twice a day for 3 days. Rats were allowed post-operative recovery before sleep recording.

2.14.2 Sleep recording procedures

*For Paper I, s*leep was recorded during a period of 10 hours. SD rats and control rats were adapted to the sleep recording conditions during 6 hours a day for 3 to 5 days. Remaining in their home cages and having free access to food and water, rats were placed in the sound attenuated recording chambers. They were connected to a flexible recording cable by the socket, and free movement was allowed by the cable being linked to an electrical swivel fixed to a movable arm outside the chamber. Sleep was recorded using Embla equipment and Somnologica 2.0.2 software. Both SD and control rats were recorded on the same days.

Paper III. Sleep recording was conducted during a period of 8 hours in the inactive phase. Rats remained in their home cages in the colony room during sleep recording. Wireless signals from the sc telemetry transmitter were collected continuously using acquisition equipment and Dataquest ART 4.1. Both SD and control rats were recorded on the same days.

2.14.3 Sleep data analysis

For visual display and sleep scoring, different software was used in Paper I and III: Somnologica 2.0.2 software and NeuroScore 2.0.1 software, respectively. For both Papers I and III, all signals were filtered at 50 Hz to eliminate power line artefacts. The EMG signals were filtered at 5 Hz. The EEG filtering was set at 35 Hz for the low-pass filter. The high-pass filter for FF EEG was set at 3 Hz and for FP EEG it was set at 1 Hz (Paper I) or 0.5 Hz (Paper III).

In Paper I wakefulness and sleep stages were manually scored in 10 second epochs according to the criteria of Neckelmann and Ursin (1993). The following stages were scored: *Wakefulness, SWS1, SWS2*, *REM sleep* and *Transition sleep.* Scoring was performed by one experimenter and intra-rater reliability was evaluated by comparing results from the 5 first scorings to results from the 5 last scorings.

In Paper III, an automatic scoring algorithm was used on the filtered signals for 10 seconds epochs. For each rat analysis thresholds were adjusted (delta-ratio, theta-ratio, EMG-threshold, activity-threshold) for the automatic scoring to fit with the manual criteria of Ursin R. and Larsen (1983), and Neckelmann and Ursin (1993). The algorithm did not include a threshold for muscle atonia in REM sleep, leading to incorrect automatic scoring of wakefulness and REM sleep. The automatic scoring was manually re-scored with regard to REM sleep and wakefulness. Threshold for delta-ratio was normally satisfactory, thus SWS1 and SWS2 from the automatic scoring was only re-scored on a few occasions. For manual re-scoring, wakefulness, transition sleep, REM sleep, SWS1 and SWS2 were defined according to Ursin R. and Larsen (1983), and Neckelmann and Ursin (1993). The following stages were scored: *Wakefulness, SWS1, SWS2 and REM sleep* (see Paper III for more details on the criteria). Transition sleep was scored as REM sleep when there was muscle atonia

and the period was followed by REM sleep. If not, transition sleep was scored as SWS1.

An inter-rater reliability between manual and semi-automatic scoring was evaluated in 6 random recordings of 8 hours duration (a total of 2880 epochs, 10s each), giving a satisfactory mean Kappa of 0.73±0.03. The percentage agreement was 92.7% for wakefulness, 96.5% for REM sleep, 85.5% for SWS1 and 92.2% for SWS2.

The following dependent variables were computed in both Papers I and III: *Total sleep time*; *Duration of wakefulness and sleep stages*; *Sleep fragmentation* (expressed by number of episodes in wakefulness and sleep stages). *Sleep onset latency* was manually scored from recording start to stable sleep onset, the first 5 minute period of continuous sleep (Bjorvatn and Ursin 1994). *SWS2 latency* and *REM sleep latency* were scored from stable sleep onset to the occurrence of the relevant sleep stage, lasting at least two epochs.

In Paper III, EEG power spectrum distributions were investigated by Fast Fourier Transform (FFT) analyses computed offline on the unfiltered FF EEG deviations using Neuroscore software. Analyses were conducted with 10 second epochs and Hamming window overlap of 75 %. The EEG signals were visually inspected and all epochs containing movement or electrical artefacts were excluded. EEG power data were calculated for total power (0.5-60.0 Hz), non-specific to sleep or wakefulness. The EEG frequency bands characteristic for each stage were considered. Wakefulness: beta (19.5-34.5) and gamma (lower range 35.0-60.0 Hz); REM sleep: theta (5.5-9.5 Hz); SWS (SWS1 + SWS2): delta (0.5-4.5 Hz).

2.15 Statistics and data analysis

Statistical analysis was performed using Statistica (version 7.0 and 8, StatSoft, Inc). A probability level of $p<0.05$ was accepted as significant. Results were presented as mean ± S.E.M. Significant overall effects or interactions in the ANOVA analyses were further investigated by Fisher LSD post-hoc tests or by *a priori* planned comparison.

2.15.1 Paper I

Parameters of OF behaviour were assessed independently by repeated measures ANOVA (group x day). *Habituation in OF* for each activity parameter was assessed independently and analysed by Students *t*-tests for independent samples. *Number of faecal droppings* was analysed using the Mann-Whitney U test to assess difference between groups, and using Friedman ANOVA to assess differences across recording days.

Parameters of sexual behaviour were analysed using the Mann-Whitney U test to assess difference between groups, and using Friedman ANOVA to assess differences between recording days.

Sleep data were expressed and analysed as percentage change from *Day -2 (baseline), and were analysed* by repeated measures ANOVA (group x day or group x day x stage). Only *Latencies to sleep, SWS-2* and *REM sleep* were assessed independently by Students *t*-tests for independent samples to assess difference between groups, and Students *t*-tests for dependent samples to assess differences between recording days.

Correlations between *Total sleep fragmentation* and *Habituation of central activity in OF* were tested by Pearson's r, in both groups collectively and within groups.

2.15.2 Paper II

To assess the effect of novelty, the first exposure to the sucrose preference test, OF and EPM test were analysed by factorial ANOVA (treatment group x corticosterone). To assess changes over tests, sucrose preference, body weight, OF and EPM parameters were analysed by repeated measures ANOVA (treatment group x corticosterone x week) from all test days, including the first. For the ASR measure, mean values of Vmax over all 10 trials at each dB level were analysed by repeated measures ANOVA (treatment group x corticosterone x dB).

Non-parametric Friedman ANOVA's were used for analysis of defecation across days in the OF and EPM. The Kruskal-Wallis test was used for analysis of group differences on defecation.

2.15.3 Paper III

For all parameters, differences between SD and control, and between SDS, SDF and control, and between SDS and SDF were analysed.

For comparisons of behaviours of the SDS and SDF rats in the SD confrontation were data adjusted for total duration of direct exposure and compared using one-tailed t-tests.

The OF emergence test parameters were analysed between groups by the Mann-Whitney U test (SD vs. control and SDS vs. SDF) or by the Kruskal-Wallis test (SDS vs. SDF vs. control). Changes across days within groups and subgroups were analysed by Friedman ANOVA. Pre-stress levels and response levels of corticosterone were analysed separately by one-way ANOVAs.

For the ASR test, mean values over all 10 trials at each dB level were analysed for maximum response amplitude (Vmax). Decrement of Vmax was defined as the percentage change in Vmax at each dB level from the first to the last (10^{th}) trial. Preliminary Levene's test revealed significant heterogeneity of variance in Vmax scores. Raw scores were therefore square root transformed and all analyses were performed on these transformed data. Differences between groups and subgroups in Vmax and decrement of Vmax were analysed by one way ANOVA for each parameter and each dB level in separate analyses.

Sleep parameters at baseline were analysed by one-way ANOVA or by repeated measures ANOVA (group x stage). To assess changes across days (-1, 1, 14 and 21), repeated measures ANOVA were used (group x day or group x day x sleep stage). EEG power bands characteristic for wakefulness, SWS and REM sleep as well as total EEG power, were analysed in separate analyses by repeated measures ANOVA (group x day). For wakefulness (characterised with several frequency bands), 'band' was added as a repeated measure.

Chapter 3

Results

3.1 Paper I

Double SD induced altered behaviour in the OF, as SD rats showed lower central activity compared to the control rats on Day 8, 10, and 11. In contrast to the controls, SD rats showed no significant decrease in defecation over time. Like controls, SD rats showed increase in total activity in the OF over days. Regarding sexual behaviour, SD rats showed only a trend towards increased latency to ejaculate on Day 4 after SD.

Some modest changes in sleep were also induced by SD. An increase in total sleep fragmentation from baseline to Day 4 was seen, due to an increased number of SWS1 and SWS2 episodes. SD rats showed increased amount of SWS2 from baseline to Day 4. Sleep efficiency, latencies to sleep, to SWS2 and to REM sleep, and amount of REM sleep was not affected by SD.

There was a negative correlation between habituation of central activity in the OF (% change from Day 8 to11) and total sleep fragmentation on Day 4.

In short, these results show that rats exposed to SD spend less time in the central area of the OF, have no decrease in defecation over time, have more fragmented sleep and more SWS2. Behaviour in the OF was affected at most days tested (except Day 9) up to Day 11, and the strongest effect on sleep was seen Day 4 after SD. A negative correlation between habituation of central activity in the OF and

total sleep fragmentation suggests a commonality of effects of SD on both behaviour and sleep.

3.2 Paper II

A single SD induced short-term and long-term effects on behaviours typically sensitive to stress. Overall, like the controls, SD rats did not change total activity over tests in the OF, and defecation rate decreased with repeated exposure, indicating no overall effect of SD on OF behaviour. Overall in the EPM test, SD rats showed long-lasting lower activity and less time on the open arms, lower total activity, and did not become more active on the open arms across repeated exposures compared to controls. In the ASR test, SD induced a long-term effect as SD showed a higher ASR to 105 and 115 dB acoustic stimuli compared to controls. Regarding sucrose preference, SD rats showed a short-lasting reduction in preference for sucrose compared to controls, an effect that was only seen Day 2 after stress.

Behaviour was affected after both SD and IFS, however, with some similar and some different time-courses. In the OF, unlike SD rats and controls, IFS rats showed lower total activity during the first OF exposure and increased total activity over time, leading to the same level as controls on Day 21. They also showed high total defecation. In the EPM, both SD rats and IFS rats showed overall the same long-lasting altered behaviours compared to controls, i.e. lower activity and less time on the open arms, lower total activity, and did not become more active on the open arms across repeated exposures. In the sucrose preference test, SD rats and IFS rats showed the same short-lasting reduction in preference for sucrose. Unlike SD, IFS had no effect on ASR.

In line with the expectation, rats with lower corticosterone compared to rats with higher corticosterone levels, exhibited longer latency to enter the open arms of the EPM, regardless of being exposed to a stressor or not. The effect was present only at the first exposure to EPM (Day 8). Lower levels of corticosterone, but only in IFS rats, was associated with higher defecation rate in both the OF (Day 21) and the EPM (Day 8). Other measures in OF, EPM and sucrose preference test were not associated with pre-stressor levels of corticosterone. In the ASR test (Day 19), there was as

expected a somewhat higher startle response, although non-significant, in the IFS low corticosterone subgroup compared to the IFS high corticosterone subgroup. Contrary to the expectations, SD *high* corticosterone rats showed the greatest startle responses, while no effect was seen in SD rats with low corticosterone.

In short, both SD and IFS led to a short-lasting reduction in sucrose preference and long-lasting behavioural alterations in the EPM. Inescapable footshock had a greater effect on OF behaviour than did SD, but induced habituation over tests. Social defeat induced an increase in ASR seen long-term after defeat, an effect not present after IFS. Low pre-stressor corticosterone level was only associated with defecation (IFS rats) and latency to enter open arms in the EPM (all groups). The SD rats with high pre-stressor corticosterone concentration showed the greatest startle response. Thus, both 'natural' SD and 'unnatural' IFS stressors altered behaviours, with some similar and some different consequences and time-courses.

3.3 Paper III

Overall as a group, rats exposed to double SD did not show changes in sleep, EEG power, behaviour or corticosterone response to the OF emergence test or ASRs. Interestingly, contrary to the controls, SD rats did not show increased time spent in the OF arena across days.

Subgroups of SD rats, SD rats that fought back during the social confrontation (SDF) and SD rats with quick submission and passivity (SDS), showed differences in behaviour and sleep compared to each other (not compared to controls). In the OF emergence test, SDF rats showed longer latency to leave the start box on Day 23 and spent less time in the OF arena from Day 16 and throughout the experiment. They also showed more fragmentation in SWS1 and SWS2, an effect which was more robust on Day 14 and 21 after SD. The subgroups of SD rats showed non-significantly but descriptive differences in duration of wakefulness and sleep stages compared to each other (not compared to controls). Before stress, SDF rats showed more SWS2 and less SWS1, a pattern that continued throughout the experiment. On baseline only, the SDF rats showed less wakefulness. With respect to amount of REM sleep, the SDS rats showed an increase compared to their own baseline *prior* to SD

on Day 1 and 14. The SDF rats, unlike the SDS and control rats, failed to show response decrement in the startle test at the lowest sound level Day 24 after SD. Compared to control rats, SDS rats showed a strong trend to higher corticosterone response to the OF emergence test.

To sum up, no considerable overall effect of double SD on sleep or startle response was found. Only a lack of increased time spent outside the start box in the OF arena across days was observed in the SD rats. Compared to SDS rats, SDF rats showed longer latency to leave the start box, spent less time in the OF arena and showed more SWS fragmentation.

Chapter 4

General discussion

The main aim of the project was to investigate short-term and long-term effects of social defeat (SD) stress on behaviour and sleep in rats. The results show that both behaviour and sleep are affected by SD, with different time-courses of the consequences.

4.1 Consequences of social defeat

4.1.1 Consequences for behaviour in the open field (OF)

The OF test was used in all three studies, although with some differences. In Papers I and II the forced exploration OF was used, and in Paper III the free exploration OF was used.

It was expected that SD rats would avoid the more 'aversive' central sectors even more than control animals (Ramos and Mormède 1998; Ray J and Hansen 2004). Rats exposed to SD showed less central activity compared to the control rats on Days 8, 10 and 11 (Paper I), which was in line with another study (Kavushansky et al. 2009). This may reflect harm avoidance which is associated with anxiety and depression in humans (Jakšić et al. 2012; Jylha and Isometsa 2006; Young et al. 1995). The central activity was not studied on Days 7, 14 or 21 (Paper II), and central activity was not possible to assess in the free exploration OF where the start box was placed in the centre (Paper III).

Following SD, total activity was expected to be lower compared to controls, as previously shown by others (Meerlo et al. 1996a; Meerlo et al. 1996b), and which is regarded as reflecting an anxiety-like response (Ramos and Mormède 1998). Overall, no differences in total activity were shown by SD rats compared to controls when tested on Days 8 to Day 11 (Paper I) or when tested on Days 7, 14 and 21 (Paper II). With repeated exposures, SD rats showed the same behavioural pattern as their controls, which may suggest that there is no effect of SD on total activity in the OF.

Rats exposed to SD were expected to show higher defecation rate, which is often regarded as an anxiety-like response to novelty (Ramos and Mormède 1998). Compared to controls, rats exposed to SD did not show differences in defecation at any time-point. However, SD rats showed no significant decrease in defecation from Day 8 to Day 11, unlike the controls, suggesting a lack of habituation to the novel OF (Paper I), and may indicate an anxiety-like state. When tested on Days 7, 14 and 21, SD rats did not differ from controls across days, as both groups showed significant decreases in defecation, suggesting habituation (Paper II). Varying results on defecation may be due to the number of exposures to the SD (single versus double) or may be due to differences in time between OF testing (one day versus one week).

After SD, rats showed neither an increase in central sector activity nor a reduction in defecation rate over days (Day 8 to 11, Paper I), an effect that may indicate a lack of habituation to the OF, or a sustained state of anxiety. Similarly, the SD rats did not increase their time spent in the OF arena outside the start box over trials from Day 9 to Day 23, contrary to what was shown by the controls (Paper III).This lack of habituation may indicate a long-term sustained state of anxiety in the SD rats.

To sum up, SD induced anxiety-like behaviours in the OF, which were seen even long-term after defeat. SD rats showed low central sector activity, and they showed a lack of habituation of central sector activity, defecation rate and time spent in the OF arena outside the start box.

4.1.2 Consequences for behaviour in the elevated plus maze (EPM)

Behaviours in the EPM test were assessed as an indicator of anxiety (Paper II). Rats exposed to SD showed significant overall long-lasting effects on EPM behaviour

compared to controls, when tested on Days 8, 15 and 22. SD rats showed lower total activity in the EPM, which may indicate that they are less active in general, are immobile or that they freeze more, although the latter was not specifically assessed. Rats exposed to SD also spent less time and showed lower activity on the open arms, indicating that they avoid the open arms and show high harm avoidance, which may reflect anxiety-like behaviour. Defecation was not different in SD rats compared to control rats. However, defecation during a test is influenced by defecation prior to the test, which applies for all tests assessing defecation (e.g. OF and EPM). Defecation may thus be a less reliable outcome measure than behaviour.

To sum up, the results on EPM behaviour may indicate an initial and long lasting anxiety-like state in rats exposed to SD.

4.1.3 Consequences for acoustic startle response (ASR)

The effect of SD on startle response as an indicator of an anxiety-like response was studied in Papers II and III.

As expected, SD increased the ASR to 105 dB and 115 dB stimuli compared to controls on Day 19 (Paper II). Increased ASR in the SD rats is indicative of a negative affective state or higher level of anxiety as seen in humans (DSM-IV-TR, APA 2000; Lang et al. 1998). Contrary to what was expected, SD rats did not show increased ASR to any of the acoustic stimuli given on Day 24 (Paper III). The startle procedure was exactly the same for both studies (Papers II and III). However, the discrepancy in the effect of SD on ASR between the two experiments may be explained by some important procedural differences (see 4.6.3 Differences and discrepancies between studies). Interestingly, compared to the passive rats showing quick submission, rats which fought the resident during the SD confrontation before they were defeated had an absence of response decrement to 95 dB stimuli. This result will be discussed later (see 4.5 Importance of behaviour during the social defeat confrontation
- consequences for behaviour and sleep).

To sum up, a long-term increase in startle response indicated an anxiety-like state in SD rats (Paper II), however long-term effect of SD on startle response per se was also absent (Paper III).

4.1.4 Consequence for sucrose preference

The effect of SD on sucrose preference as an indicator of anhedonia was studied in Paper II.

A transient reduction in preference for sucrose was observed in SD rats compared to controls, an effect apparent only on the first test Day 2 after SD. The short duration of the effect may indicate that the anhedonic effect is not a depression-like symptom, as the effect then would be expected to last longer. The reduced sucrose preference may indicate anxiety, which is also associated with anhedonia (Der-Avakian and Markou 2012; Grillo 2012).

The use of sucrose in the test for anhedonia is a debated topic. Both sucrose and saccharine consumption have been used as indicators of anhedonia. The underlying motivation for sucrose intake may be both for caloric intake and for hedonic reasons, while saccharin is non-caloric and has mainly hedonic properties. However, in addition to the hedonic property of the sweet palatable taste, saccharin has a bitter, aversive taste, which in itself may induce reduced intake at certain concentrations (Dess 1992). In this respect sucrose may be a better choice, even if it has caloric properties.

To sum up, SD caused a transient reduction in preference for sucrose, an effect that may be interpreted as an anhedonic state. The effect is not believed to be a depression-like symptom, as the effect is only seen Day 2 after defeat, but may be a transient anxiety-like symptom.

4.1.5 Consequences for sexual behaviour

The effects of SD on sexual behaviour, as an indicator of anxiety-like and/or depression-like behaviour, were studied in Paper I.

Contrary to what was expected, no effects were seen Day 1 and only a trend towards increased latency to ejaculate on Day 4 after SD was shown. Reduction in sexual behaviour has been shown after several exposures to SD in rodents (Niikura et al. 2002; Yoshimura and Kimura 1991), while the effect on sexual behaviour was studied after double SD in the present study. Thus, a higher number of exposures to SD than used in the present project may be needed to induce effects on sexual behaviour in rats. Another possibility is that altered sexual behaviour following

double SD may need more time to develop as no effect was found on Day 1 and a trend towards increased latency to ejaculate was seen Day 4. The effect of SD on sexual behaviour was only evaluated until Day 4, which may have been a too short time for any alteration of sexual behaviour to manifest itself. Testing of sexual behaviour a longer time after SD should be considered in future studies.

To sum up, sexual behaviours in SD rats were marginally affected, as only a trend to increased latency to ejaculate was seen Day 4 after defeat. Thus, there was no evidence of an anxiety-like or depression-like reduction in sexual behaviour.

4.1.6 Consequences for sleep

Possible sleep alterations as an effect of SD were studied in Papers I and III.

In line with the hypothesis, SD caused an increase in total sleep fragmentation from baseline to Day 4 (Paper I), due to a higher number of SWS1 and SWS2 episodes in the SD group. Within the SD group, an increased number of SWS2 episodes were seen across days. Sleep fragmentation represents a problem of maintaining sleep which may be seen in patients with anxiety (Mellman et al. 1995) and depression (Kupfer 1995). Increased fragmentation of sleep may be induced by hyperarousal due to activation of the stress response system (Chrousos and Gold 1992). The fragmented sleep present on Days 1 and 4 after SD may thus indicate hyperarousal and an anxiety-like and/or depression-like state.

Rats exposed to SD also showed a negative correlation between total sleep fragmentation on Day 4 and habituation of central activity in the OF. Even though this is based on a low number of animals, the data suggest that the most harm avoidant animals had the highest sleep fragmentation, and less harm avoidant animals showed lower sleep fragmentation. This may indicate a commonality of effects of SD on both behaviour and sleep, and may be interpreted to be an anxiety-like and/or depression-like effect on behaviour and sleep.

A reduced amount of deep SWS is seen in depressed patients (Peterson and Benca 2011), while results in anxiety patients are inconclusive (Kobayashi et al. 2007; Papadimitriou and Linkowski 2005). Social defeat induced an *increase* in SWS2 from baseline to Day 4, but no effect was seen Day 1 (Paper I). This short-term consequence may indicate an anxiety-like sleep rather than a depression-like

sleep as it was not seen long-term after SD (Paper III). The increase in SWS2 on Day 4 may possibly reflect a re-occurring need for deep sleep after SD, as stress alters sleep homeostasis.

The sleep stage SWS2 is comparable to SWA, because more than 50 % of the 10 seconds epoch scored contains SWA (Papers I and III). Slow wave activity refers to delta, amplitude and power and requires neither a minimum amplitude nor a percentage of the epoch criterion, as is the case for scoring of deep SWS.

Increased SWS after stress may be explained by the synaptic homeostasis hypothesis by Tononi and Cirelli (2006), which predicts that the higher amount of synaptic potentiation in cortical circuits during wakefulness, the higher the increase in SWA during subsequent sleep, and may indicate a higher synaptic potentiation during SD stress. The function of this increase in SWA is hypothesized to promote downscaling of net synaptic strength, which benefits e.g. learning and memory (Tononi and Cirelli 2006). The essential function of sleep is according to their hypothesis the restoration of synaptic homeostasis. Another hypothesis of sleep homeostasis is that increased metabolic activity in the brain and body during wakefulness is accompanied by an increased rate of metabolite synthesis higher than the rate of clearance, resulting in increased levels of metabolites. When a critical level is reached, metabolic processes respond by moderating the wake-active neuronal systems, and sleep may be induced. Sleep reduces the synthesis of metabolites (Datta and Maclean 2007). It has recently been shown that the extracellular space in the cortex increase during sleep, and increased flow of cerebrospinal fluid gives a rapid clearance of toxins and metabolites (Xie L et al. 2013), an additional explanation of the restorative effect of sleep.

Sleep recording on Day 1 did not reveal any effect on SWS2 (Papers I and III). An increased amount of SWA has previously been seen acutely after SD (Meerlo et al. 1997). This acute effect of SD might have occurred also in the present studies, but could have disappeared during the hours between SD and sleep recording, as rats were allowed to sleep during this period.

Increased amount of REM sleep and shortened REM sleep latency are common features of depression (Palagini et al. 2013; Peterson and Benca 2011). After

SD, no change in the amount of REM sleep or latency to REM sleep was found at any time-point, indicating a lack of common depression-like sleep alterations. However, changes in duration and latency to REM sleep are not consistent findings in PTSD and GAD, as these patients have shown both shorter and longer REM latency, higher and shorter duration of REM sleep, and also no difference compared to control subjects (Papadimitriou and Linkowski 2005). Thus, the absence of REM sleep changes in the present project might be an indicator of anxiety-like sleep or normal REM sleep, but possibly not depression-like sleep.

Increased REM density is associated with PTSD (Kobayashi et al. 2007) and depression (Palagini et al. 2013), and has to my knowledge not previously been reported in animal models of stress. However, a study on fear-conditioned rats showed increased neck muscle twitches during REM sleep (Madan et al. 2008). The effect was reasoned to correlate to bursts of eye movements in REM sleep, and to be consistent with several human studies on PTSD. Hence, in future preclinical studies on sleep after stress it would be useful to measure REM density by studying neck muscle twitches as a correlate to eye movements in REM sleep.

Sleep and sexual activity were tested at the same time-points (in different animals, Paper I). That SD affected sleep but not sexual activity at Day 4 may indicate different time-courses of manifestation of altered sleep and sexual behaviour.

Contrary to what was expected, when all animals were included as one experimental group in Paper III, there were no effects of SD on sleep fragmentation or amount of SWS2 (as found in Paper I), neither short-term nor long-term, and no other sleep alterations associated with anxiety and/or depression were found. The absence of effects on sleep in the SD rats as one group, in Paper III compared to Paper I, may be due to differences in experimental procedures which will be discussed below (see 4.6.3 Differences and discrepancies between studies). Another explanation may be the timing of sleep recording. In Paper III, there were no results on sleep between Day 1 and Day 14, as a large part of the data from the sleep recording Day 7 were lost and the remaining data were not analysed. In between these time-points sleep changes may have appeared and disappeared, and the effect

seen on Day 4 (Paper I) may thus have been missed in Paper III. More studies are needed to reveal the time-course of the development of sleep alterations after SD.

To sum up, rats exposed to SD showed modest changes in sleep. They showed high sleep fragmentation, due to increased number of SWS1 and SWS2 episodes. The effect was evident on Day 4 after SD, and may have been due to a higher arousal level in the SD rats. High sleep fragmentation was associated with avoidance of the central sector of the OF. The SD rats showed an *increase* in SWS2 from baseline to Day 4, possibly indicating a re-occurring sleep need. Overall as a group, there was no *long-term* effect on sleep in rats exposed to SD. The results of the present study may indicate that sleep in the rats exposed to SD is more weighted towards an anxiety-like state, as the effects were not long lasting and sleep alterations typically seen in depressed patients were not present.

4.2 Comparing the effects of social defeat and inescapable footshock

The effects of SD stress were compared with that of inescapable footshock (IFS) stress, recognized as an animal model of anxiety, on behaviours typically sensitive to stress (Paper II). Similar effects in SD and IFS rats were expected.

As previously discussed, the SD rats generally did not differ from the control rats in the OF test, which suggests no effect of SD in this particular behavioural test. Unlike SD rats and controls, IFS rats showed lower total activity during the first OF exposure, but increased their total activity with repeated exposures, leading to the same level as controls on Day 21. Compared to SD and control rats, IFS rats showed high total defecation. Taken together, this suggests initial anxiety-like and/or depression-like behaviour in the IFS rats, an effect that gradually fades and is no longer present by Day 21. As the IFS model is regarded as an animal model of anxiety, the effect may represent a state of anxiety and not a depression-like state.

Since the same rats were repeatedly tested in Paper II, any modulation over tests could reflect either a temporal dissipation of the effects of IFS, or habituation to the test situation, or both. A previous study found that IFS rats showed lower locomotion in an OF on Days 1, 2, 4, 7 and 14, and increased defecation on Days 4, 7 and 14 (Van Dijken et al. 1992c). In that study, contrary to Paper II, OF testing was

performed on independent groups of rats for each day to prevent any habituation. If IFS rats in the present study did not habituate to the OF test over days, they would possibly show the same low locomotion on all days of testing as shown in the study of van Dijken et al. This may indicate that IFS rats in the present study habituated to the OF test, and that the modulation over tests was not a temporal dissipation. To study the exact time-course of the behavioural response to stress, independent groups of rats must be tested to prevent effects of habituation.

In sum, SD and IFS induced different effects on OF behaviour, as IFS rats showed lower total activity. The effect gradually disappeared with repeated exposures, indicating habituation. The results may suggest a higher initial anxiety-like state in the IFS rats as shown by OF behaviour.

Both IFS and SD rats showed initial and persistent high anxiety-like behaviour in the EPM as measured by less time and lower activity on the open arms compared to controls, as well as lower total activity. This is in line with previous studies on the effects of IFS (Kavushansky et al. 2009; Korte et al. 1999; Louvart et al. 2005). With the design of re-exposures to EPM chosen for the present study, both SD and IFS rats show similar anxiety-like behaviours.

Contrary to what was expected, only rats exposed to SD and not IFS rats showed increased ASR to 105 dB and 115 dB acoustic stimuli. This result on the IFS rats was thus not in line with a previous finding (Milde et al. 2003). In the present project the same ASR and IFS procedures were used as in the study of Milde and colleagues. However, there were some differences in the IFS apparatus used and the experimental design that may explain the lack of effect of IFS on ASR in the present study. In the present study, rats were placed individually in the apparatus for IFS induction. The previous study used an apparatus where the individual shock chambers were smaller (partially restraining) and IFS rats had more olfactory and auditory contact, compared to the present apparatus (for details, see Paper II and Milde et al. 2003). The results may indicate that the apparatus in the previous study was the most anxiety inducing. Regarding the experimental design, rats were not exposed to any behavioural testing between IFS and the ASR test in the previous study, as they were in Paper II. Another methodological difference was housing conditions. These

differences in experimental design and housing conditions will be discussed below (see 4.6.3 Differences and discrepancies between studies). It was not an aim of the present study, but to reproduce the Milde et al. study, a simpler experimental design must be used, with no behavioural tests between stress and startle testing, and with more similar housing conditions. In short, single SD and IFS differed in the effect on startle response, as only SD rats showed an increased startle response, indicating high anxiety in only SD rats.

In the sucrose preference test, IFS rats like the SD rats showed a temporary reduction in preference for sucrose compared to controls. The stress-induced anhedonic behaviour was apparent only on the first test, applied two days after the stressors. A similar transient change in preference for sweet solutions (saccharin) was reported by Van Dijken et al. (1992a) using an IFS protocol similar to the one used in the present study. Thus, single SD and IFS showed a similar transient decrease in preference for sucrose, an effect that may be interpreted as an anhedonic state.

In a previous study, the effects of SD and IFS have been compared to controls on behaviour in the OF and EPM (Kavushansky et al. 2009). Lower total activity in both tests and a lower open arm activity in the EPM were seen in IFS rats but not in SD rats. The OF result was in line with the present study, but not in line with the anxiety-like behaviour shown in the EPM by both IFS and SD rats in the present study. The authors concluded that the stressors differ in their behavioural outcome, which is in line with the overall picture seen in the present study.

To sum up, the results strongly indicate that the behavioural effects of single SD and IFS differ, as concluded by others (Kavushansky et al. 2009), even if the differences are not revealed in all of the measures chosen. The two stressors have similar effects on sucrose preference and EPM behaviours, while they differ with respect to the effects on OF behaviours and startle responses. These results may reflect fundamental differences between the two stressors used. The difference in consequences may be due to the quality of the stressor, ‘natural’ versus ‘un-natural’ or there may be differences in controllability of the stressor. In the defeat situation SD rats can to some extent control the attack from the resident by submitting, while the IFS rats have no controllability over the footshock exposure. From this study it is

not possible to claim that the two stressors are equal in intensity. Such a claim would require physiological measures of the stress response to each stressor.

4.3 Is social defeat an animal model of anxiety or depression, or both?

Anxiety and depression are complex disorders with separate diagnoses in the diagnostic systems used today (DSM-IV-TR, APA 2000; DSM-5, APA 2013; ICD-10, WHO 2008). However, it is in many cases difficult to clearly separate anxiety from depression in patients due to the frequent comorbidity and overlapping symptoms and signs, e.g. harm avoidance behaviour, sleep alterations, and sexual impairment. Thus it is equally difficult to claim that social defeat is an animal model of either anxiety or depression.

The present project showed that SD induced low activity in the central sector of the OF from Day 8 to Day 11, indicating high harm avoidance which has been associated with anxiety and depression in humans (Jakšić et al. 2012; Jylha and Isometsa 2006; Young et al. 1995). Also, a lack of habituation was seen across days as the SD rats did not show increased central activity or decreased defecation rate from Day 8 to 11. Similarly they did not increase their time spent in the OF arena outside the start box from Day 9 to 23. This long-term lack of habituation may reflect an anxiety-like state. SD also induced a short-lasting reduced preference for sucrose, high startle responses on Day 19, and in the EPM test long-lasting lower total activity, less time and lower activity on the open arms. These altered behaviours may be interpreted more as anxiety-like behaviours.

After exposure to SD, rats showed a short-term effect on sleep, i.e. increased sleep fragmentation and increased duration of SWS2 from baseline to Day 4, but no long-term effect was seen. The lack of long-term sleep alterations, lack of effect on REM sleep (duration and latency) and lack of reduction in SWS2 may indicate that the sleep alterations found are more directed towards anxiety-like sleep.

Previously single exposure to SD has been described as an animal model of depression (Carnevali et al. 2012; Kavushansky et al. 2009; Koolhaas et al. 1990). The present project however shows that the alterations induced by single and double SD are collectively more directed towards an anxiety-like state, rather than

depression. However, many of the alterations studied and found (e.g. locomotor activity, harm avoidance, sexual behaviour and sleep) may be associated with both anxiety and depression. Additionally, the only test that was aimed to test a symptom most typically associated with depression (anhedonia - core symptom of depression) was the sucrose preference test. Future studies should also include tests more specific to depression-like behaviour (e.g. the forced swim test), to possibly add to the conclusion of the present project that single and double SD effects are more directed towards anxiety-like effects. Also, the predictive validity of single and double SD would be necessary to confirm such a distinction. Behavioural effects of single SD have been shown to be reversed by Clomipramine, Fluoxetine (antidepressant and anxiolytic drugs), by a CRH antagonist and by the acutely anxiety reducing agent midazolam (Berton et al. 1999; Heinrichs et al. 1992; Koolhaas et al. 1990). So far, there is not a clear picture of the predictive validity of single SD.

In chronic SD, where animals are daily exposed to SD over weeks, more depression-like symptoms are induced, which are reversed by antidepressant drugs and not by anxiolytics (Iio et al. 2012; Rygula et al. 2006; Rygula et al. 2005; Rygula et al. 2008). Such a chronic protocol should be used in future studies if the aim is to study depression-like symptoms in the animals.

4.4 Importance of the pre-stressor levels of corticosterone for the effects on post-stressor behaviour

The relationship between levels of corticosterone prior to the SD or the IFS stressor and the different post-stressor behaviours was explored (Paper II).

It was expected that lower pre-stress levels of corticosterone would be associated with high startle responses in the IFS rats, in line with the previous study of the research group (Milde et al. 2003). The same was expected for SD rats. Such a relationship was not found, neither in IFS rats or in SD rats. However, what is interesting is that the direction of the non-significant differences between means of startle in the IFS and control subgroups are as before, with higher ASR associated with lower initial corticosterone in IFS and control rats (Figure 5, Paper II). Also contrary to the expectations was the finding that those *SD* rats with *higher* pre-stress

corticosterone concentration showed the highest ASRs of all. Amongst rats with lower corticosterone, SD did not enhance the startle response. Thus, the pattern of the higher and lower corticosterone SD rats is the opposite from the higher and lower corticosterone IFS and control rats.

The present study showed that rats with lower pre-stress corticosterone concentrations exhibited increased latency to enter the open arms of the EPM than rats with higher corticosterone levels, regardless of whether they had been exposed to a stressor or not (Table III, Paper II). This supports the hypothesis that that low corticosterone has implications for anxiety-like behaviours.

Greater defecation rate in both the EPM and the OF was also associated with lower levels of pre-stressor corticosterone (Table II, Paper II). However, there was a difference between stressors. IFS rats with lower corticosterone showed more defecation than SD rats with lower corticosterone on the first exposure to the EPM and the third exposure to the OF. These findings indicate that lower pre-stress corticosterone level may be a vulnerability factor for the anxiety-like effects of IFS, but not of SD stress, when defecation is used as the measure. However, other measures in the OF, in the EPM and in the sucrose preference test were not associated with lower or higher pre-stressor levels of corticosterone.

To sum up, the relationship between levels of corticosterone prior to the SD or IFS stressor and the different post-stressor behaviours was not as expected. Lower pre-stressor corticosterone level was only associated with defecation in IFS rats, and latency to enter open arms in the EPM in all groups. Opposite to what was expected, the rats with *higher* pre-stressor corticosterone concentration that were exposed to SD showed the greatest startle response. These results may also reflect fundamental differences between the SD and the IFS stressor.

4.5 Importance of behaviour during the social defeat confrontation - consequences for behaviour and sleep

Throughout the project, large variations were observed in the behaviours displayed by the intruders when confronted by the resident. In Paper III, the SD rats were split into two subgroups on the basis of this behaviour: intruders that showed no

resistance (SD submissive, SDS) and intruders that fought back during one or both of the SD confrontations (SD fighters, SDF). It should be emphasised that all SD rats were eventually defeated. Previous studies have shown that SDS rats show the greatest short-term and long-term alterations (Meerlo et al. 1999; Stefanski 1998; Walker et al. 2008; Walker et al. 2009; Wood SK et al. 2010; Wood SK et al. 2013). This was also expected in Paper III.

Contrary to what was expected for OF behaviour, SDF rats exhibited a longer latency to leave the start box. They did not increase time spent in the arena across days and on Days 16 and 21 after SD they spent significantly less time in the arena. The result may indicate higher harm avoidance in the SDF rats, which may be associated with anxiety and depression in humans (Jakšić et al. 2012; Jylha and Isometsa 2006; Young et al. 1995). An alternative explanation for the behaviour in the OF emergence test is behavioural flexibility versus rigidity. The observations may fit in with the ideas of Koolhaas and his group (Benus et al. 1991b; Koolhaas et al. 2010; Koolhaas et al. 1999). Over a series of studies in a number of species, they have observed that aggression is associated with some rigidity of routines (intrinsically driven behaviours, proactive coping), while non-aggression is more associated with flexibility of behaviours (extrinsically driven behaviours, reactive coping). In the present study, the SDF animals' unwillingness to leave the start box might reflect greater rigidity of behaviour, while the SDS rats' greater willingness to leave the start box and to explore the open arena might reflect greater flexibility of behaviour. Behaviours shown by the SDF rats in the OF may thus indicate more harm avoidance and/or greater rigidity of behaviour.

In line with what was expected, the SDS rats had a close to significantly higher corticosterone response to the third OF emergence test, compared to control rats. Descriptively, SDS rats had higher corticosterone responses than SDF rats, which again had higher corticosterone responses than controls. Earlier studies have shown a similar difference in corticosterone response between rats with different behaviour displayed during the SD confrontation, as passivity in the SD confrontation was associated with a high corticosterone response (Walker et al. 2009). Furthermore, high resting levels of corticosterone are seen in rats with short submission latency

before the fourth confrontation in a series of 7 SD confrontations on consecutive days (Wood SK et al. 2010). However, it has been suggested that glucocorticoids participate to the mediation of reward, counteracting the aversive effects of external aggressions, allowing more adaptive responses to threatening situations (Piazza and Le Moal 1997). As the SDS rats show the highest corticosterone response to OF, their response to the SD confrontation may also have been higher, allowing them to adapt to the SD. The corticosterone response to the SD should therefore be assessed in future studies to examine this hypothesis. Measurement of corticosterone levels acutely after SD was excluded in the present studies as the sampling method required anaesthesia which may interfere with e.g. sleep architecture as seen in humans (Moote and Knill 1988). It should also be noted that there was no difference between subgroups or difference with controls regarding pre-stress corticosterone levels.

Startle response amplitude to acoustic stimuli did not differ between the subgroups of SD rats or between subgroups and controls. However, unexpectedly, SDF rats showed a lack of decrement of the startle response to the 95 dB stimulus intensity, compared to the SDS and control rats. The term habituation is not used for this response decrement since the stimuli were presented at three intensities in a pseudo-random order. It is however worth noting that a lack of habituation of the startle response magnitude and skin conductance has been reported in PTSD patients (Jovanovic et al. 2009; Metzger et al. 1999). The absence of response decrement in SDF animals may therefore reflect an increased state of anxiety.

Different sleep patterns were found in the two subgroups of SD rats. Contrary to what was expected, the SWS continuity was significantly poorer (greater fragmentation) at the end of the experiment in the *SDF rats* compared to the SDS rats. In both SWS1 and SWS2, the SDF rats showed a more consistent architecture of sleep throughout the experiment with a significantly higher fragmentation. The SDS rats seemed to decrease their fragmentation across days.

The two subgroups of SD animals interestingly showed different, however not significant, patterns in the various sleep stages *prior* to and after SD. SDF rats were less awake, showed less SWS1, more SWS2, and more REM sleep. This pattern of differences in SWS1 and SWS2 was maintained from baseline throughout the

experiment, suggesting that this might reflect a trait rather than an effect of social conflict. An interesting link to the higher amount of SWS2 in SDF rats is that high slow-wave EEG power is found in humans with aggressive behaviour, and has been interpreted as impaired inhibitory control and/or cortical immaturity (Bronsard and Bartolomei 2013). As a higher amount of SWS2 was present in SDF rats even before SD stress, this may be a predictor for how they behave in the SD confrontation and a vulnerability factor for anxiety-like and/or rigid behaviour in the OF.

With respect to amount of REM sleep, the SDS group showed an increase compared to their own baseline prior to SD on Day 1 and Day 14. This result for the SDS group, although not significant, is interesting because a disinhibition of REM sleep is a predictive marker of depression (Steiger and Kimura 2010).

Previous studies showed that rats opposing the resident during the social conflict seem less affected by the defeat than those who show quick submission and passivity. Therefore it is a paradox that the results to some extent suggest that rapid submission during a social confrontation might be more adaptive than fighting back. SDF animals had more sleep fragmentation, greater rigidity of behaviour and/or anxiety in the emergence test, and a lack of decrement in startle response over repeated trials. Such a response pattern is similar to that described as ‘proactive’ by Koolhaas and his group (Benus et al. 1991b; Koolhaas et al. 2010; Koolhaas et al. 1999). However, it must be recalled that all animals eventually lost their fight.

Submissive behaviour after a shorter fight may be more adaptive, as the resident normally stops the attack at this signal, injuries are avoided, and the ‘physical attack’ period of the SD confrontation is ended. The SDF rats were in confrontation with the resident for a longer time, they had a lack of controllability and predictability as they could not escape, and the resident was giving mixed signals by lying on its back, but at the same time being the aggressive one. This may be why the SDF rats seemed more affected in the present study.

To sum up, contrary to the expectations, SDF rats showed more sleep fragmentation, more rigidity of behaviour and/or anxiety-like behaviour in the OF emergence test, and a lack of startle response decrement. This suggests that surrender after a short fight may be more adaptive than surrender after a longer fight.

4.6 Methodological considerations

4.6.1 Animals

The strain of experimental rats in this project was the Wistar strain, derived from *rattus norwegicus,* which have been widely used to study the effects of stress (e.g. Milde et al. 2003; Rygula et al. 2005; van Dijken et al. 1992a). The Wistar strain is an outbred strain, with large genetic variation, chosen to mimic the large variation in the general human population. In humans, only a minority develop affective disorders after exposure to stress, and this may be explained by e.g. individual differences in genetics, previous experience to stress, their coping style or personality. Such individual differences are also present in animals. Using animals with a large genetic variation may result in anxiety-like and/or depression-like behaviour in only some of the animals, but at the same time reflecting the diversity in humans. A small number of outbred rats may give too large a variance within both control and stress exposed groups, reducing the possibility of revealing significant effects. To increase the effect in future studies, a larger number of animals may be needed, and individual differences may be controlled for by grouping animals according to their phenotype (e.g. specific coping style or pre-stress behaviour), or strains bred for lower genetic variability may be used.

Outbred animals were separated into subgroups before stress on the basis of corticosterone (Paper II). The rats with low pre-stress corticosterone level were expected to show the most prominent anxiety-like behaviours following stress. The hypothesis was not supported by the data. Also, animals were separated into subgroups on the basis on how they behaved in the SD confrontation (Paper III). This lead to some interesting results, and is recommended in future studies. Other possibilities are to subgroup rats on the basis of behaviour prior to stress, to use inbred rats with lower genetic variability or to use animals that are genetically manipulated. Commercial strains of rodents with targeted disruption of the serotonin transporter (5-HTT) (Murphy et al. 2001) may be useful, as 5-HTT promoter polymorphism is associated with anxiety and depression in humans (Caspi et al. 2003; Xie P et al. 2009). Using chosen subgroups or specific strains of rodents may result in lower variance and yield more significant effects.

4.6.2 The social defeat conflict

As other authors have also acknowledged, a social interaction is not easy to standardize (Meerlo et al. 2001). Even if the resident is aggressive in the training session, the new intruders in the SD conflict may have other strategies and coping styles, making the conflicts differ both quantitatively and qualitatively. In all SD confrontations, the resident rat was the one that showed dominant behaviour. However, some of the resident BD IX rats showed primarily dominant but occasional submissive (supine posture) behaviours. Subsequently, more dominant behaviour was displayed by the resident and eventually the SD rat showed submissive behaviour. This might have led to a qualitatively different defeat compared to the rats that were immediately defeated. However, when the SD rats fought back, they were in the confrontation with the resident over a longer period before they were separated after defeat, which may be the reason why they seemed to show the largest effect of SD.

Submissive behaviour, as shown by lying on the back, usually results in the resident stopping the attack. Not all Wistar rats clearly demonstrate this submissive behaviour, which have also been observed by others (Professor Jaap Koolhaas, personal communication, 15th May, 2006). In the present studies, the Wistar rats however showed other clear submissive behaviours like flight, freezing, defensive-upright position, and some also showed offensive behaviours like moving toward the resident, initiating attacks, and holding the resident down (see Appendix for Description of behaviours and postures displayed in a social confrontation between two male rats). In 2013, a paper on standardizing the resident-intruder paradigm was published (Koolhaas et al. 2013). The authors recommend to videotape and record the full behavioural repertoire of the experimental animal during the test to allow an unbiased analysis of the results. The duration of the confrontation should be fixed to solve the quantitative aspect, but the confrontations can still be qualitatively different. Analyses of the intruders' behaviours during the confrontation will give a qualitative evaluation of the social interaction. The protocol suggested by Koolhaas et al. (2013) is recommended for future studies to increase the standardization of studies on SD stress.

4.6.3 Differences and discrepancies between studies

There were some discrepancies across experiments in the effects of SD procedures on behaviour and sleep. The discrepancies may be due to differences in experimental procedures. Five differences in particular warrant attention.

Firstly, the rats in Paper I were housed in conventional cages while the rats in Papers II and III were housed in individually ventilated cages (IVC), as demanded by more stringent ventilation requirements. Little is known about the effect of IVC on behaviour, and the confounding factor that this environmental change may represent. Housing in IVC involves a greater degree of isolation than does conventional housing. Isolation and housing in IVC are both suggested to increase anxiety (Logge et al. 2013; Pritchard et al. 2013). Our research group has recently studied the effects of individual housing in IVC versus conventional cages (Jellestad et al. 2013). Rats housed in IVC showed less weight gain and lower OF activity, suggesting higher anxiety. In the present project, individual housing in IVC may therefore have induced anxiety-like behaviour in both control rats and stress exposed rats, possible reducing the differences between the groups.

Secondly, one study did not include surgery (Paper II), while the surgery for sleep recording differed significantly between the studies (Papers I and II). Surgical procedures for Paper III were considerably more invasive than those for Paper I and required a longer post-operative resting period (see 2.14.1 Surgical procedures above). Also, some of the rats implanted with the subcutaneous transmitter needed more post-operative care, and consequently they were more handled. This led to differences in amount of handling between studies. It has been shown that handling may have an anxiety reducing effect in rats (Costa et al. 2012; Schmitt and Hiemke 1998). Thus, in Paper III the higher amount of handling of those rats needing more post-operative care may have had an anxiety reducing effect in these animals. This again may have increased the variation between animals, possible limiting the effects of SD on sleep fragmentation.

Thirdly, the effects of SD on OF behaviour and sleep were assessed in different papers, but at different time-points and with different intervals between measurements. Differences in time-points after SD when the effects on behaviour or

sleep are measured may lead to different results. The reasons for this possibly lie in that the stress response changes over time, and that effects of stress have different time-courses and may appear not acutely but over time (Koolhaas et al. 1997b). In this regard, it was an advantage for the project that behavioural testing was done at different time intervals to get a picture of both short-term and long-term effects of SD.

Fourthly, single and double exposure to SD was chosen for the project, but it was not an aim of the study to directly compare the two protocols. A previous study has shown that single and double SD induce similar effects in some behavioural aspects, but other behaviours are more affected by double SD (Meerlo et al. 1996b). As in that study, the present studies show that different aspects of behaviour are differentially affected by single and double SD with respect to the magnitude and time-course of the changes induced. Differences in numbers of exposure to the SD (single versus double exposure in our studies) may have caused divergent results, as the rat may become sensitized to subsequent stressors (Koolhaas et al. 1997a).

Fifthly, behavioural tests may influence performance during consequent behavioural tests. Notably, OF and EPM are anxiety provoking behavioural tests which may serve as stressors in themselves, and rats may become sensitized to subsequent stressors (Koolhaas et al. 1997a). This may possibly explain the weakening of the initial IFS-induced anxiety-like behaviour in the OF, and the lack of effect of IFS on startle response (Paper II). Also controls have been exposed to the anxiety-inducing behavioural tests, which might have increased their ASR to some extent and decreased the difference between controls and IFS rats. However, it is unclear why the intervening behavioural tests should not also have weakened the SD-induced increase in startle response in Paper II.

To sum up, difference in housing conditions, surgical impact, timing of effect assessment, number of stress exposures and influence of consequent behavioural testing are all factors that may have contributed to differences in the results between studies in the project. On the other hand, it was not in the aim of the project to directly compare studies.

4.6.4 Ethical considerations

In animal studies ethical guiding principles are the three R's of Replacement, Reduction and Refinement (Russell and Burch 1959).

Replacement is the substitution of living animals to alternative techniques like in vitro techniques (cells and tissues), computerized models, etc. To date there are no alternatives to live animals in experimental models of affective disorders. Thus, replacement was not possible in the present study.

Reduction of animals was taken into consideration as re-exposures to behavioural tests were used in the present project. In this way, behaviour at different time-points was tested in the same rats, not utilizing new rats for each time-point as others (Meerlo et al. 1996a; Van Dijken et al. 1992c). With re-exposure of rats to behavioural tests, one must bear in mind that this design may induce habituation. To study habituation in itself may be an advantage, as healthy persons and patients with affective disorders may show differences in habituation. Regarding the residents and females used in the project, the number could have been reduced. New residents and females were used for each study in the present project. When a research group is planning to use the SD model, it is recommended that several studies are planned in advanced to allow for repeated use of the residents and their female cage mates.

Refinement was assessed as the blood sampling method was less invasive in the present project, originally with collection from the jugular vein in the rat's neck, later with collection from the saphenous vein in the rat's hind limb. There were used different sleep recording methods in the project. In Paper I, the rat was connected to a freely moving cable for sleep recording. In Paper III, sleep was recorded in undisturbed animals in their home cages through wireless telemetry. The latter sleep recording method may thus be considered a refinement. This refinement in sleep recording must however be weighed up against more invasive surgical procedures. Refinement of the surgical procedures must be continued e.g. by using smaller transmitters for implantation.

4.7 Future research

Based on the discussion in this thesis, some issues for future research should be outlined.

Among individuals experiencing stress, only a sub-population will develop a disorder. Thus, individual differences and vulnerability factors for development of psychopathology must be further investigated. In the present project, low pre-stress corticosterone level was investigated as a possible vulnerability factor. However, a clear association with anxiety-like behaviour was not found. The separation of SD rats with proactive and reactive coping strategy during SD may provide the possibility to study coping style as a vulnerability factor. In future studies it would be useful to screen animals in a behavioural test before the SD stress to reveal which animals will be the 'proactive' copers. The defensive burying test, where proactive copers bury more (De Boer and Koolhaas 2003; Koolhaas et al. 2010), might be a useful tool for making this pre-stress subdivision. Alternatively, stereotypic behaviours induced by apomorphine may be used to discriminate between coping styles (Benus et al. 1991a).

The present study only investigated face validity of the SD model. It would be worth investigating the predictive validity of the model by testing the effects of known anxiolytics and/or antidepressants to decrease the effects induced by the stress regime, as found by others (Berton et al. 1999; Heinrichs et al. 1992; Koolhaas et al. 1990).

The effects of SD on sleep should be further investigated. Sleep fragmentation was found Day 4 after SD, an effect not present Day 14 after SD. Between these time-points other sleep alterations may develop and this should be further investigated to identify the time-course of the development of sleep alterations following SD. After SD, increased SWS2 was found in the present study and increased SWA in previous studies (Meerlo et al. 2001; Meerlo et al. 1997). Increased SWS2 as a consequence of SD stress may be used as a model to further study the synaptic homeostasis hypothesis and the restorative effect of increased SWS2/SWA. Also more specific, quality of sleep associated with affective disorders (e.g. REM density) should be included in future studies on animal models of affective disorders.

Additionally some methodological issues have been discussed. The standardized protocol suggested by Koolhaas et al. (2013), by e.g. using a fixed duration in physical contact with the resident, should be considered in future studies. The amount of handling and housing conditions should also be taken into consideration since these may have impact on the outcome measures and not least hinder replicability. And last, in line with ethical guidelines, the residents and their female cage mates should be used in several studies on SD to reduce the number of animals.

4.8 Summary and conclusions

In conclusion, the studies presented in this thesis show that exposure to SD induced both short-term and long-term consequences for multiple behavioural features and at least short-term consequences for sleep. These effects generally support the SD model as having a high degree of face value as a model for affective disorders. The intention was to evaluate if SD can reproduce the alterations in locomotor activity, harm avoidance, startle response, anhedonia, sexual behaviour and sleep, parallel to those observed in humans with anxiety and/or depression.

Social defeat induced low activity in the central sector of the OF, indicating high harm avoidance, which may reflect anxiety-like or depression-like behaviour. No short-term or long-term effects were seen on total activity in the OF. Further, a lack of habituation was seen across days on central activity, defecation rate (Day 8 to 11), and time spent in the OF arena outside the start box (Day 9 to 23). This long-term lack of habituation may again reflect heightened anxiety. Overall, in the EPM test, SD rats showed less total activity, less percentage time and less activity on the open arms, indicating high harm avoidance. Effects in the EPM test were interpreted as long-lasting anxiety-like behaviours. High startle response was seen as a long-term effect of SD and may reflect an anxiety-like state. A short-lasting reduced preference for sucrose was seen after SD, which may indicate an anhedonic state, and may be interpreted as a transient anxiety-like symptom. Sexual behaviour was not affected in the present study.

The SD rats showed an increase in sleep fragmentation and SWS2 duration from baseline to Day 4 after SD. As a group, SD rats did not show long-term effects on sleep or EEG power. The effects of SD on sleep may be interpreted as anxiety, because common sleep alterations in depression were not induced (e.g. reduced deep SWS and REM sleep alterations).

When comparing the effects of SD stress with the effect of IFS stress, the two stressors had similar short-term effects on sucrose preference and long-term anxiety-like behaviours in the EPM test. However, IFS rats showed lower total activity in the OF test, while the SD rats showed the highest startle response. The results may reflect fundamental differences between SD and IFS.

Opposite to what was expected, the rats with high pre-stressor corticosterone concentration that were exposed to SD showed the greatest startle response, while low pre-stress corticosterone level IFS rats did not show alterations in startle response. This may further support fundamental differences between the SD and the IFS stressor.

Two subgroups of rats with different coping styles in the SD confrontation showed dissimilar effects on sleep and behaviour in the OF emergence test. Contrary to what was expected, rats fighting back in the SD confrontation showed longer latency to leave the start box and spent less time in the OF arena compared to SDS rats, indicative of anxiety-like and/or depression-like behaviour. They also showed more fragmentation of sleep in SWS1 and SWS2. The results may suggest that rapid submission during defeat may be more adaptive than surrender after a longer fight given these outcome measures.

Overall, the results of the present study indicate that social defeat leads to complex changes in behaviour and sleep that are relevant for studies of affective disorders in humans. Both the behavioural (EPM, ASR and OF) and sleep changes (fragmentation) observed overall suggest that single or double exposure to SD might be more relevant to anxiety than to depression, but tests of predictive validity would be necessary to confirm such a distinction.

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