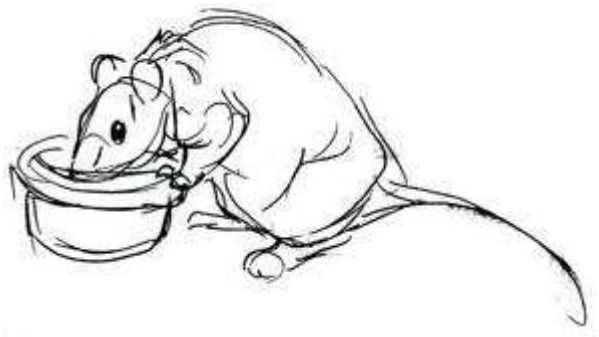
CHRONIC MILD STRESS - AN ANIMAL MODEL OF DEPRESSION

FROM BEHAVIOR TO MOLECULES



Janne Grønli

Thesis for the degree Philosophiae Doctor (PhD)
Department of Biomedicine, Section of Physiology,
University of Bergen, Norway
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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamin, serotonin
5-HIAA	5-hydroxyindolacetic acid
aCSF	artificial cerebral spinal fluid
ANOVA	analysis of variance
BDNF	brain derived neurotrophic factor
BSA	albumin from bovine serum
cAMP	cyclic adenosine monophosphate
CMS	chronic mild stress
CREB	calcium/cyclic-AMP responsive binding
DA	dopamine
DRN	dorsal raphe nucleus
ECL	enhanced chemiluminescence
ECS	electroconvulsive shock
EEG	electroencephalogram
EMG	electromyogram
FF	fronto-frontal
FP	fronto-parietal
FST	forced swim test
GABA	γ-aminobutyric acid
HPA	hypothalamic-pituitary-adrenal
HPLC	high performance liquid chromatography
HVA	homovanilic acid
ICSS	intracranial self stimulation
LDT	laterodorsal tegmental
LSD	least significant deviation
LTP	long term potentiation
MAOI	monoamine oxidase inhibitor
MDD	major depressive disorder
NA	noradrenaline
NAc	nucleus accumbens
NMDA	N-methyl-D-aspartate
p-CREB	phosphorylated CREB
PPT	pedunculopontine
REM	rapid eye movement
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPD	Sprague-Dawley
SSRI	selective serotonin reuptake inhibitor
SWS	slow wave sleep
TBST	tris-buffered saline/tween-20
TCA	tricyclic antidepressant
VTA	ventral tegmental area
W	waking

LIST OF ORIGINAL PAPERS

This doctoral thesis is based on the following papers.
They are referred to by their roman numerals in the text.

I. Grønli, J., Murison, R., Bjorvatn, B., Sørensen, E., Portas, C.M., Ursin, R.

Chronic mild stress affects sucrose intake and sleep in rats.
Behavioural Brain Research, 150 (2004) 139-147.

II. Grønli, J., Murison, R., Fiske, E., Bjorvatn, B., Sørensen, E., Portas, C.M., Ursin, R.

Effects of chronic mild stress on sexual behavior, locomotor activity and consumption of sucrose and saccharine solutions.
Physiology & Behavior, 84 (2005) 571-577.

III. Grønli, J., Bramham, C., Murison, R., Kanhema, T., Fiske, E., Bjorvatn, B., Ursin, R., Portas, C.M.

Chronic mild stress, an animal model of depression, inhibits BDNF expression and CREB activation in the dentate gyrus
Submitted, 2006.

IV. Grønli, J., Murison, R., Fiske, E., Bjorvatn, B., Sørensen, E., Portas, C.M., Ursin, R.

Effects of chronic mild stress on hippocampal extracellular 5-HT levels in sleep an wakefulness.
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1. INTRODUCTION

1.1 General aspects of depression

Disorders of mood, or affect, have been described since the 4th century BC. Despite this early acknowledgment, their aetiology is still a source of debate. There is a growing knowledge that, far from being a disease with purely psychological manifestations, depression is now known as a complex disorder involving the whole body and the diagnosis of depression is based on a heterogeneous set of symptoms. The criteria have gradually developed as documented by both the American Psychiatric Association (Diagnostic and Statistical Manual of Mental Disorders, DSM-IV, 1994) and the World Health Organisation Geneva (International Classification of Diseases, ICD-10, 1993), providing essential guidance for both clinicians and researchers.

According to DSM-IV, a major depressive disorder (MDD) manifests with symptoms at the psychological, behavioral and physiological levels. An episode requires the presence for at least 2 weeks of one or two core symptoms: dysphoric mood and anhedonia (a loss of interest or pleasure in activities that usually would be enjoyed). In addition, four of the following symptoms must be present (three if both core symptoms are present): disturbances of sleep, feelings of worthlessness or guilt, inability to concentrate or think, increased or decreased psychomotor activity, decreased sexual drive, appetite disturbance or weight change and suicidal thoughts.

Major depression is a common disorder, and the prevalence is increasing. The World Health Organization estimates that by 2020 unipolar major depression will become the second largest cause of global disease problems in the world, only behind ischemic heart disease (Murray 2001). Therefore, depression represents a major medical and social problem. The lifetime prevalence for major depression is reported to be as high as 14-17 % and the 12 month prevalence is 4-8 % (Kessler 2003; Alonso et al. 2004). A Norwegian epidemiological study reports the prevalence in Oslo residents to be 17% and 8%, respectively (Kringlen *et al.* 2001). Mood disorders tend to appear in the third decade of life, but the first occurrence of major depression can be at any point in life.

1.2 Hypotheses of depression

No one knows the precise mechanism that triggers clinical depression. In the early 20th century the explanation of mental illness changed from a disease of the 'mind' to a proper brain dysfunction. Today, neuroscientists know that, in many cases, psychopathology arises as a consequence of altered regulation of particular brain chemicals. In addition, cell and molecular biology have proved useful to study the mechanisms of information processing, plasticity and neuronal survival involved in mood disorders.

1.2.1 Monoamines and depression

After the serendipitous discovery of the antidepressant effects of monoamine treatment during the 1950s, the first biochemical hypothesis of depression was formulated in the mid 1960s (Bunney and Davis 1965; Schildkraut 1965). The main assumption of this hypothesis is that clinical depression is due to impairment of central monoaminergic function, a deficiency in the neurotransmission mediated by serotonin (5-HT, 5-hydroxytryptamin), norepinephrine (NA) and dopamine (DA). The monoamine concentrations may be altered as a result of disrupted synthesis, storage or release, or the concentrations may be normal but the postsynaptic receptors and/or sub-cellular messenger activity may be impaired. Hence, the treatment of depression is supposed to increase the availability of the amines in the brain. Different mechanisms may increase the availability of brain monoamines. These include blocking the reuptake of the monoamine in the synapse, inhibiting the intraneuronal metabolism of the monoamine or blocking the presynaptic inhibitory auto- or heteroreceptors (Blier and Ward 2003; Hensler 2003). Monoamines affect a wide range of functions central in depression like sleep, vigilance, appetite, motivation, motor activity and reward and their imbalance may produce symptoms like aggression, euphoria and impulsiveness. Loss of interest or pleasure in activities that are normally pleasurable is one of the core symptoms of depression. The brain dopaminergic system is crucially involved in reward behavior and/or motivation, especially the meso-limbic projections to the nucleus accumbens (NAc) and prefrontal cortex (Wise 1978; Phillips 1984). A reduced plasma concentration of homovanilic acid (HVA), a dopamine metabolite, is found in depressed patients (Lambert et al. 2000).

Research on the first antidepressants, monoamine oxidase inhibitors (MAOI) and tricyclic antidepressants (TCA) demonstrated their ability to facilitate noradrenergic and/or serotonergic neurotransmission, which correlated with behavioral excitation. However, the non-specific action of TCAs reuptake inhibition leads to a range of undesirable side effects. Both preclinical and clinical studies have clearly shown that selective reuptake inhibitors, e.g. acting on a single monoamine system, give a therapeutic advance. With the introduction of the selective serotonin reuptake inhibitors (SSRIs) in the mid-1970s, a 5-HT-related hypothesis gained significance. Today, the SSRIs are the most commonly prescribed antidepressants. However, monoamine depletion in healthy individuals (control patients) does not consistently produce depressive symptoms. Tryptophan (precursor of 5-HT) depletion does not affect mood in healthy subjects, but does change mood in subjects with a history of psychiatric illness (Benkelfat et al. 1994; Carpenter et al. 1998; Knott et al. 1999). In addition, tryptophan depletion produces alterations in rapid eye movement (REM) sleep typical of depression [15] whereas relapse into depression produced contrasting results in patients treated with SSRIs (Delgado et al. 1991; Bremner et al. 1997; Nutt et al. 1999; Evans et al. 2002). In healthy animals, tryptophan depletion is known to increase pain sensitivity, motor activity and aggression (Young et al. 1988; Kawai et al. 1994). A microdialysis study showed a reduction in the 5-HT release after tryptophan depletion only in animals previously treated with the antidepressant fluvoxamine (Bel and Artigas 1996). Both serotonergic and noradrenergic compounds are useful in treating depressive patients. Some dopaminergic drugs have also been successfully in the treatment of depression (Slattery et al. 2004). After depletion of tyrosine (a precursor of dopamine) the performance of healthy volunteers in specific neuropsychological tests paralleled that of unipolar depressed patients (McLean et al. 2004). However, a rapid elevation of monoamines is not correlated with quick antidepressant action (Heninger et al. 1996). Other brain chemicals may be involved in depression like neurokinins, γ-aminobutyric acid (GABA), glutamate, neuroactive steroids, opioids, cholecystokinin, histamine and nicotine (see review: (Slattery *et al.* 2004)). There is not clear evidence for one transmitter being central to the aetiology of depression. The complex multifaceted nature of depression is made up of a variety of emotional, behavioral and cognitive elements. It is possible that each of these components of the syndrome may involve different neurobiological substrates (Asberg et al. 1976).

1.2.2 Neuronal plasticity, information processing, neurogenesis and depression

One emerging hypothesis suggests that problems in information processing within specific neural networks, rather than changes in chemical balance, might bring out depression. Depression may arise when some neuronal systems do not exhibit appropriate, adaptive plasticity in response to external stimuli. This possibility is supported by the fact that most antidepressant drugs induce plastic changes in neuronal connectivity, which gradually lead to improvements in neuronal information processing and recovery of mood (Duman et al. 1997; Duman et al. 2000; Hashimoto et al. 2004). Regulations of intracellular messenger cascades mediate the ability of neuronal systems to adapt in response to pharmacological and environmental stimuli (Duman et al. 1994). A broad division classifies the intracellular signal transduction pathways into two categories, those that are regulated by G-protein receptors coupled second messengers (e.g. cAMP, Ca^{2+}, etc) and those that are regulated by receptors coupled directly or having a close interaction with protein tyrosin kinases (Manji *et al.* 1995). The first category is controlled by neurotransmitters (monoamines and neuropeptides), the second by cytokines and growth factors (including the neurotrophin family, e.g. brain-derived neurotrophic factor (BDNF)). Hence, regaining the plasticity within these molecular cascades is likely to contribute to the effects of the antidepressants.

BDNF is the most widespread growth factor in the brain, responsible, among the other functions, of neuronal survival. To activate transcription of BDNF a phosphorylation of calcium/cyclic-AMP responsive binding protein (CREB) at its transcriptional regulatory residue Serine-133 is necessary (Tao et al. 1998; Conti et al. 2002). Levels of serum BDNF are decreased in major depressive patients (Karege et al. 2002) and are associated with vulnerability to develop mood disorders in healthy subjects (Lang et al. 2004). A common finding in animal studies is that, when administrated chronically, almost every antidepressant elevates CREB and its activated form, phosphorylated-CREB, in the hippocampus and cerebral cortex (Takahashi et al. 1999; Jensen et al. 2000; Thome et al. 2000). In line with this finding, increased CREB levels have been reported in postmortem brains of antidepressant-treated subjects (Dowlatshahi et al. 1998). Contrary to this, an increase in CREB and p-CREB is found in drug-free depressed suicide victims (Odagaki et al. 2001). In rats, both chronic antidepressant treatments and electroconvulsive shock (ECS) increase BDNF levels (Nibuya et al. 1995; Russo-Neustadt et al. 1999).

Administration of BDNF displays antidepressant effects in two animal models of depression; the forced swim test (FST) and the learned helplessness model (Siuciak et al. 1997).

It has been suggested that a reduced hippocampal cell number may be involved in the pathophysiology of depression (Malberg 2004) and treatment with antidepressants has been shown to increase hippocampal neurogenesis (see review: (Malberg and Schechter 2005)). Clinically, there is evidence of reduced hippocampal volume in patients with MDD or other affective disorders (Sheline et al. 1996; Sheline 2000). A stress-induced decrease in dentate gyrus neurogenesis may be critically involved in the development of depressive episodes (Duman and Charney 1999; Jacobs et al. 2000; Gage 2002). Changes in neurogenesis do occur after acute stress (McEwen 2000), however, reduced cell proliferation in the dentate gyrus does not appear to be correlated to the development of learned helplessness (Vollmayr et al. 2003; Henn and Vollmayr 2004).

1.3 Stress and affective disorders

Hans Selye coined the term ‘stressor’ in 1950 to differentiate the condition of stress from the stimulus which evokes it. In the beginning of the ‘30s, Selye initiated a pioneering program of studies on the adaptive physiology of stress which set the standard for modern biological research in this area (Selye 1949; Selye 1956). Current theories distinguish four different aspects of the term ‘stress’; stress stimulus, stress experience, stress response and the experience of the stress response (Ursin and Eriksen 2004). The occurrence of stress is described by basic phenomena that are the same across cultures and species (Eriksen et al. 2005). A stress response starts with the central nervous system processing sensory information related to external stimuli. When a particular situation is interpreted as potentially harmful, a complex cascade of neural, hormonal and behavioral responses is initiated to cope with the situation. Stress related alterations in neuronal and neuroendocrine processes generate an alarm response and general arousal. Briefly, this response includes increased attention to the surroundings, accelerated heart rate, increases in blood pressures, increases in respiration rate and alterations in metabolic processes. Stress as a functional response is necessary for successful coping with environmental challenges. It enables an

organism to deal with the wide variety of aversive and undesirable situations present in everyday life facilitating survival in a continuously changing environment (Ursin and Olff 1993). However, responses vary according to individual stress sensitivity (e.g. genetic or due to previous experience) and the type of stressors, whether it is acutely or chronically presented, whether it its controllable or uncontrollable.

The way animals, including humans, cope with stressful events show considerable individual variation. This variation may underlie a differential vulnerability to develop stress-related pathologies. Stress experience has been suggested to have a direct or indirect causal association in the development of depression (Anisman and Zacharko 1982; Brown 1993). Most people subjected to stressful events do not develop depression. However, depressed patients often have a history of stressful and traumatic experiences. A manifestation or worsening of depression in adulthood is often related to acute life events or ongoing stress (Hammen et al. 1992). Depressed individuals often report that they feel helpless, hopeless, or unable to control events, and consequently exposure to uncontrollable stressful events has been hypothesized to be significant in bringing about depression (Seligman et al. 1980).

Depression is often associated with physiological changes characteristic of a normal stress response. There has been a major focus on the role of the hypothalamic-pituitary-adrenal (HPA) axis as a marker of the stress response. Stress response activates the HPA axis and the release of glucocorticoids, which increases the heart rate, blood pressure, and metabolism. A consistent finding in depressed patients is hyperactivity and dysregulation of the HPA-axis, demonstrated by increased cortisol levels, enlargement of the pituitary and adrenal glands and decreased glucocorticoid receptor sensitivity (Sachar 1970; Krishnan et al. 1991; O'Toole et al. 1997; O'Toole et al. 1998). Also, the HPA axis is found to be a mediator of changes in brain monoamines, e.g. locus coeruleus noradrenaline (Mello Ade *et al.* 2003) and raphe serotonin (Chaouloff 2000). Antidepressants, both individually and in conjunction with anti-glucocorticoid agents, reverse a number of the HPA-axis abnormalities (Anand et al. 1995).

Other stress-regulated brain chemicals, e.g. certain cytokines that affect the HPA axis, may induce depressive-like syndromes in individuals without a history of mood disorders (Connor and Leonard 1998; Maier and Watkins 1998). Childbirth provokes secretion of cytokines, which have been postulated to contribute to postnatal

depression (Maes et al. 2001). Cytokines also affect monoaminergic transmission (Hindmarch 2001).

1.4 Sleep in affective disorders

Sleep alterations is associated with affective disorders (Oswald *et al.* 1963; Zung *et al.* 1964; Gresham *et al.* 1965; Hawkins and Mendels 1966). The most common complaint of sleep disturbance in patients with major depression is insomnia. Difficulty falling asleep, frequent nocturnal awakenings, early morning awakening, non-restorative sleep, decreased total sleep, and disturbing dreams with more negative emotional content are often reported.

Objective sleep disturbances as assessed by polysomnographic recordings, confirm subjective experience in the majority of depressed patients. However, manifestation of most sleep abnormalities only occurs when depressive symptoms are present. Usually, sleep alterations in depression are grouped into three general categories (see review: (Benca et al. 1992)):

1) Sleep continuity disturbances. Prolonged sleep latency, frequent arousals during sleep and early awakening in the morning. The sleep is more fragmentated which results in decreased sleep efficiency and reduced amount of sleep (Oswald *et al.* 1963; Zung *et al.* 1964; Gresham *et al.* 1965; Kupfer *et al.* 1973).

2) Slow wave sleep (SWS) changes. SWS processes appear to be abnormal in depression as indicated by a reduction of the SWS (Hawkins and Mendels 1966; Kupfer and Foster 1973). In general, SWS loss is more significant during the first part of the night usually containing more SWS (Kupfer et al. 1985; Kupfer et al. 1986). However, not all groups of depressed patients display significant loss of SWS in comparison to controls (Kupfer and Reynolds 1989, Quitkin et al. 1985, Thase et al. 1989).

3) REM sleep changes. A reduced REM sleep latency (period of time from sleep onset to the first REM sleep period), prolonged duration of the first REM sleep episode, increased percentage of REM sleep and more frequent eye movements (increased REM sleep density) during REM sleep are often reported in depression.

Another strong link between mood disorders and sleep is suggested by the observations that depressive symptoms are improved by sleep deprivation and reoccur

after sleeping. A variety of sleep manipulations has been shown to have a rapid antidepressant effect. They include selective deprivation of REM sleep, partial and total sleep deprivation. More than 60 % of depressed patients report improved mood following one night of total sleep deprivation, and almost 90% after three sessions performed at one-week interval (see reviews (Wu and Bunney 1990; Wirz-Justice and Van den Hoofdakker 1999)). However, the effect is short-lasting, and in most cases relapse occurs after the first episode of recovery sleep (Southmayd et al. 1990).

The frequent coexistence of mood alterations and sleep abnormalities suggests that a common pathogenesis may be present. The observations of REM sleep alterations together with the REM sleep-suppressing capabilities of most antidepressants suggest that there is an enhanced REM sleep "pressure" in depressed patients (Vogel 1983). Most antidepressants enhance central monoaminergic activity, especially serotonergic activity. Serotonin modulates sleep and wakefulness (see review: (Ursin 2002)). The electrophysiological activity of dorsal raphe nucleus (DRN) serotonergic neurons and the level of extracellular 5-HT at somato-dendritic and terminal levels show a maximum during wakefulness, a decrease during SWS and a minimum during REM sleep (for review see: (Portas et al. 2000)). In addition, serotonin directly modulates the activity of REM sleep promoting neurons in the pedunculopontine (PPT) and the laterodorsal tegmental (LDT) nuclei (Sanford et al. 1994). Since REM sleep is selectively impaired in depression, it is possible that the decreased serotonergic function hypothesized in depression, may produce a disinhibition of the REM promoting areas and a consequent increase of REM sleep. This may also explain why prolonged wakefulness appears to have a favorable effect in depressed patients. A prolonged wakefulness may induce acute mood improvement by enhancing serotonergic neurotransmission (perhaps similarly to SSRIs). Following the same reasoning, it has been hypothesized that the symptom of insomnia, frequent in depressed patients, may be therapeutical rather than pathological, an attempt to counteract the serotonergic hypofunction (Adrien 2002). In line with this hypothesis, chronic sleep loss in rats has been found to cause persistent desensitization in the serotonergic autoreceptors 5-HT_{1A} (Roman et al. 2005). A desensitization of these receptors would induce an increased firing of serotonergic neurons due to the lack of negative feed-back.

1.5 Emotionality and altered activity in affective disorders

Depressed mood, feelings of worthlessness, inappropriate guilt and suicidal thoughts are some of the most frequently reported affective changes in depressed patients. These symptoms of increased emotionality are mainly described verbally by the subjects. However, changes in emotionality can, at some degree, be reflected in presence of abnormal activity. The changes are often seen as psychomotor agitation, slowness of motor activity or alteration in novelty-induced behavior reflecting anxiety, fatigue or increased harm avoidance. In DSM-IV, psychomotor agitation is defined as 'excessive motor activity associated with a feeling of inner tension'. Psychomotor retardation, slowness of movement, is nearly an opposite motor disturbance that can be among the earliest symptoms of depression. The important brain regions that regulate locomotor activity, the striatum and the cerebellum, receive extensive monoaminergic input. Serotonergic and dopaminergic nuclei project to both, whereas noradrenergic nuclei project to the cerebellum only. An impairment of these neurotransmitter systems may be the basis of the locomotor alterations observed in depressed patients. Novelty seeking is a tendency toward frequent exploratory activity and intense excitement in response to novel stimuli. Patients suffering from depression have been reported not to differ in the novelty seeking, but have an increased harm avoidance compared to healthy people (Kusunoki et al. 2000). Harm avoidance is an intense response to aversive stimuli, to avoid punishment and novelty, possibly reflecting increased feelings of vulnerability, fear and anxiety. In addition, emotionality has been shown to correlate with an increased ambulatory blood pressure and heart rate. Emotional individuals show higher levels of perceived daily stress, trait anxiety and depressive symptoms (Carels et al. 2000). These symptoms can be objectively measured in animal models of depression e.g. using an open field test to measure changes in locomotor activity, novelty-induced and exploratory behavior and harm avoidance (Denenberg 1969; Walsh and Cummins 1976).

A diminished motivation is a core symptom of clinical depression that can be manifested by alterations of sexual function (Shabsigh et al. 2001). Similarly, rodents subjected to procedures that induce a depressive-like state exhibit impairment of sexual drive (i.e., latency and frequency of mounts, intromission and ejaculation) (Neill et al. 1990; D'Aquila et al. 1994; Vogel et al. 1996). Decrease in the number of

ejaculations and intromission frequency are considered as signs of weakening of sexual activity in rodents (Serra G 1988).

2 Animal models of depression

Animal models of depression have the purpose to reproduce some known aspects of depression in selected animal species (e.g. rodents). On this basis they can be used: 1) as a tool for investigating aspects of the neurobiology and pathophysiology of depression; 2) as experimental models for studying the mechanism of action of antidepressant drugs; and 3) as screening tests for elucidating antidepressant activity. Antidepressant drugs have little or no effect in healthy individuals. The number of validated animal models for affective disorders is large and still growing. In addition, several minor variations have been applied to each model. A summary of the models can be found in several reviews: (Willner 1997; Cryan et al. 2002; Nestler et al. 2002; Shaffery et al. 2003)

2.1 Validation criteria

The problem in all animal models, and especially models for psychiatric conditions which are in part defined through subjective experience, is to define clear criteria that allow asserting the validity of the model. McKinney and Bunney (McKinney and Bunney 1969) proposed more than 30 years ago that the minimum requirements for an animal model of depression are: 1) there is a ‘reasonable analogy’ to the human disorder in its manifestations or symptomatology; 2) there is a behavioral change that can be monitored objectively; 3) the behavioral changes observed should be reversed by the same treatment modalities that are effective in humans; and 4) the model should be reproducible between investigators. A major problem in depression research is the lack of validated models. Many of the symptoms of depression (e.g. depressed mood, feelings of worthlessness, suicidality) cannot easily be measured in laboratory animals.

Willner (Willner 1984) refined McKinney and Bunney criteria (McKinney and Bunney 1969) and proposed different types of validity: construct validity, face validity and predictive validity.

Construct validity addresses the theoretical rationale of the model. However, this evaluation largely relies on the present knowledge of the pathophysiology of depression, which is far from being fully understood. In addition, it is very difficult to take into account the multiple factors involved in the development of depression like psychological (e.g. stressful life events, adverse experiences, personality traits) or biological factors (e.g. genetic influences, physical illnesses, medications).

Face validity refers to the similarity between the behavior modeled in the animal and the symptoms of depression. A model which parallels multiple symptoms of human depression is considered valuable.

Predictive validity concerns the extent to which the model responds appropriately to antidepressant effect as in humans. A valid model should be sensitive and specific, that is, it should respond to effective antidepressants but not to non-selective drugs and the responses should occur within an appropriate dose range.

2.2 Stress exposure in models of human depression

Stressful life events are environmental factors that may play a role in the etiology of depression (see section 1.3). Thus, a valuable model of depression could result from an altered behavioral state induced by stress response. It is assumed that exposure to uncontrollable stressors induces a feeling of loss of control might result in a depressive behavioral state.

Learned helplessness. This paradigm was originally described in dogs by Overmier and Seligman (Overmier and Seligman 1967). Animals are exposed to inescapable electrical shocks. When unable to avoid the repeated aversive stimuli they renounce to escape (even when this possibility is available). The model has been found to be reproducible in many species including rodents and appears to mirror many aspects of human depression. The helpless animals show weight loss, agitated locomotor behavior, sleep changes, decreased libido, decreased learning, alterations in the HPA axis, anhedonia (e.g. increased threshold for intra-cranial self stimulation (Zacharko

and Anisman 1991) and lower intake of sucrose or saccharin compared to control animals (Minor *et al.* 1994; Vollmayr *et al.* 2003)). Some investigators report that the effect of helplessness is limited to 3 days, others report maintenance of the helpless behavior for 10 to 14 days (Vollmayr and Henn 2001; Vollmayr *et al.* 2003). The effects can be reversed after treatment with a variety of antidepressants, ECS and even cognitive training (Overmier 1968; Sherman and Petty 1982). However, not all animals exposed to inescapable shocks develop helplessness. This suggests that individual sensitivity to stress is an important factor to develop depression, as observed in human depression.

Chronic stress. The first chronic stress model of depression was developed by Katz (Katz 1981). In this model, various stress procedures which involve relatively harsh stressors (electrical shock, 40h food deprivation, cold swim, water deprivation, heat stress, shaker stress etc.) were applied. The animals were exposed to unpredictable stress for a 21-day period. Not all animals survived this regime. Those which survived, were found to exhibit anhedonic responses expressed as a decreased intake of sucrose and saccharine, decreased locomotor activity and elevated corticosterone levels. MAOIs and amitryptiline restored normal behavior. Today there is a wide range of chronic models of stress based on harsh stressors, mild stressors or a combination of the two. Stressed animals are supposed to develop 'anhedonia' as inferred from a reduction in sucrose consumption, as well as variety of cardiovascular or neuroendocrine consequences. These effects are reversed by long-term antidepressant treatment (Willner 1997). Considering face and construct validities, chronic stress models appear more suitable for the experimental investigation of depression compared to acute stress models. This may be due to the fact that chronic frustration and chronic stress are more likely to induce neurobiological changes leading to depression. The major disadvantage of these models is the poor reproducibility in behavioral abnormalities and their response to antidepressants within and between laboratories (for review: (Willner 1997).

Early life stress. Several models involving manipulation of the early life environment have been used, including 'prenatal stress', 'early postnatal handling' and 'maternal separation'. The early life stress models produce neuroendocrine and behavioral changes that persist into adulthood and make the animals sensitive to stress in later

life. For example, animals subjected to early stress show a hyperactive HPA axis, alterations in neurotransmitter systems, increased responses to novelty and greater vulnerability for learned helplessness and drug self-administration. Usually the resulting abnormalities can be reversed by antidepressant treatment, although negative findings have also been reported (for review: (Nemeroff and Vale 2005)).

2.3 Chronic mild stress (CMS) in rats

In 1987, Willner and co-workers developed the chronic mild stress (CMS) protocol which includes a variety of low-grade stressors administered over a long period of time (Willner et al. 1987). The presentation of different stressors is an essential feature of the model, as repeated presentation of a single stressor results in rapid behavioral habituation (Muscat and Willner 1992). The CMS regime includes soiled cage, tilting of the cage, alterations of the light-dark cycle, periods of food or water deprivation, grouping etc (Willner *et al.* 1987). The animals are exposed to each stressor for a short time (few hours to a day) over several (2-5) weeks. This model better reflects the human situation characterized more by daily hassles than traumatic events. CMS results in some behavioral abnormalities that parallel symptoms observed in human depression and different antidepressants reverse these symptoms. Willner and collaborators originally reported that CMS induces anhedonic behavior, one of the core symptoms of major depression. This adds credits to the CMS as being a reliable model of depression in accordance to the three main validation criteria (see review (Willner 1997; Willner 2005)).

2.3.1 CMS induced anhedonia

Anhedonia, meaning 'without (*an*) pleasure (*hedonia*)', can be defined as 'the diminished capacity to experience pleasure of any sort'. It may reflect a personality trait or it may be a specific state or symptom in various psychiatric disorders (DSM-IV, ICD-10, (Loas 1996)). One of the most salient symptoms of a major depressive episode is the failure to obtain pleasure from activities that previously were enjoyed (e.g. recreational activities, sex, eating).

How do we measure animals' pleasure? How do we get animals to loose their feeling of pleasure? Such assessment is based on assumptions since we cannot communicate verbally with the animals. Katz (Katz 1982) found that exposure to chronic severe stress produced reductions in sucrose and saccharin consumption. Willner and co-workers (Willner *et al.* 1987) subsequently claimed that such stress induces appetitive changes resembling the anhedonic changes observed during episodes of human depression and would, therefore, serve as an ideal measure with which to monitor the effect of CMS in rodents. Hence, the hedonic measure of a natural reward for palatable solutions is the primary validation measure of the CMS model. When given a choice, rats, like most humans normally prefer to drink sweetened liquids. CMS produces a significant reduction in the sucrose consumption. This effect persists for up to 8 weeks (Willner *et al.* 1987) and is reversed by tricyclic antidepressants. Decreased place preference conditioning (Papp et al. 1991; Valverde et al. 1997), and higher brain stimulation thresholds (i.e. intra cranial self stimulation (ICSS) (Moreau et al. 1992; Moreau et al. 1994; Moreau 1997)) also suggest decreased response to rewarding stimuli after CMS. It has been demonstrated that CMS rats show a lower number of Fos-positive neurons in the key area of the dopaminergic reward system, NAc, compared to Controls (Grippo et al. 2004).

2.3.2 CMS induced behavioral and physiological changes

In addition to anhedonic behaviour, chronic mild stress produces a wide variety of symptoms that parallel many features of human depression and providing strong face validity to the model. CMS has shown to decrease aggressive and male sexual behavior in rats (D'Aquila *et al.* 1994). Furthermore, the animals show decreased locomotor activity during the active phase (Gorka et al. 1996), altered circadian and diurnal rhythm (Gorka *et al.* 1996; D'Aquila *et al.* 1997), and sleep changes including an abnormal REM sleep pattern (Moreau et al. 1995; Cheeta et al. 1997). Moreover, CMS results in a relative loss of bodyweight (Willner et al. 1996), an altered sympathetic cardiac regulations (Grippo et al. 2002; Grippo et al. 2003) and an altered level of cytokines (Grippo *et al.* 2003; Li *et al.* 2003; Grippo *et al.* 2005). CMS also affects the HPA axis activity (e.g. adrenal hypertrophy, Muscat and Willner 1992 and/or corticosterone hypersecretion (Ayensu et al. 1995). Altogether, the model generates both behavioral and physiological abnormalities characteristic of human

depression. CMS does not seem to cause anxiety (as may be expected after acute or severe stress administration), supporting the specificity of depression-relevant behavior (D'Aquila *et al.* 1994).

The model is pharmacologically sensitive and a variety of antidepressant treatments, including ECS, are effective in reversing anhedonic behaviour in CMS rats. Among the first substances found to be effective were the TCAs (Willner *et al.* 1987; Moreau *et al.* 1992; Sampson *et al.* 1992; Papp *et al.* 1996) and the SSRIs (Muscat et al. 1992; Przegalinski et al. 1995; Marona-Lewicka and Nichols 1997; Willner 1997). Also the atypical antidepressant mianserin (Moreau *et al.* 1994), MAOs (Moreau *et al.* 1993; Papp *et al.* 1996) and the ECS (Moreau *et al.* 1995) are effective. The antimanic drug lithium (Papp *et al.* 1996), the 5-HT_{1A} agonist buspirone, the corticosterone synthesis inhibitor ketoconazole (Przegalinski *et al.* 1995) and competitive NMDA antagonists (Papp and Moryl 1994) also show antidepressant activity in the model. Chronic treatment with antidepressant drugs appears to affect specifically the behavior of CMS animals, leaving controls unaffected, as in humans (Willner *et al.* 1992; D'Aquila *et al.* 1994; Willner 1997). However, response to non-selective antidepressants has been reported after antihistaminergic and anticholinergic drugs (Papp *et al.* 1996). Chlordiazepoxide (anxiolytic), D-amphetamine (psychostimulant), neuroleptics and α-2 antagonist ethoxyidazoxan are reported to be ineffective (Moreau *et al.* 1992; Willner *et al.* 1992).

2.3.3 Neurobiological consequences of CMS

There are few reports about the neurobiological consequences of CMS. Recently, a reduced cortical amount of serotonin has been reported after CMS (Li *et al.* 2003; Bekris *et al.* 2005). An altered function of the serotonergic system is suggested by the findings of 5-HT_{2C} hypersensitivity (Moreau et al. 1996) and by an upregulation of cortical 5-HT_2 receptors, an effect that is reversed by imipramine (Klimek and Papp 1994).

The hippocampus is a key structure for studying the neurobiological substrates of stress and depression (McKittrick et al. 1995; Graeff et al. 1996), due to its involvement in the regulation of the HPA axis (De Kloet et al. 1998) and the high number of corticosteroid and 5-HT receptors (Uphouse 1997). Studies on the effect of

CMS on the serotonergic activity in hippocampus have shown inconsistent results. In vivo, the level of hippocampal extracellular 5-HT is decreased (Kang et al. 2005), while in the homogenized brain tissue an increase (Bekris *et al.* 2005), a decrease (Li *et al.* 2003) and no change (Haidkind et al. 2003) are described. The binding to hippocampal 5-HT_{1A} receptors is increased by CMS and further increased by imipramine treatment (Papp *et al.* 1994).

There are discrepancies in the findings of dopaminergic activity after CMS. Di Chiara and collaborators (Di Chiara and Tanda 1997) found that CMS did not affect basal DA level in the NAc or the prefrontal cortex, whereas Bekris et al. (Bekris *et al.* 2005) found an increased dopamine amount in the homogenized tissue of the prefrontal cortex and a decreased amount in the striatum. Di Chiara argues that CMS affects the responsiveness of the dopaminergic system to motivational stimuli, facilitating a stimulatory DA response to aversive stimuli but blunting stimulatory responses to rewarding stimuli (Di Chiara and Tanda 1997). A change in the dopaminergic function in the NAc (involved in the CMS-induced anhedonia) is also suggested by receptor binding studies (Grippo *et al.* 2004). A decreased number of DA D2 receptors, rather than a change in receptor affinity, is found in the limbic forebrain (but not in the striatum). Such decrease is reversed by imipramine treatment (Papp et al. 1994; Dziedzicka-Wasylewska et al. 1997). Other findings suggest that an increased sensitivity of postsynaptic DA receptors is responsible for the reversal of anhedonia (e.g. normalized sucrose intake after antidepressant treatment). Such normalization reverses into anhedonia after blockage of the D_1 or D_2 receptors (Muscat *et al.* 1992; Sampson *et al.* 1992).

A microdialysis study of the basal extracellular levels of [Met]enkephalin-like material in the rostral part of the NAc showed similar levels in CMS and Control group. Exposure of the two groups to social interaction increased the extracellular level in the Controls but not in the CMS rats (Bertrand et al. 1997). This suggests that the reactivity of the endogenous opioid system could be reduced after CMS. Neuropeptides such as neuropeptide Y, Substance P and galanin are also altered by CMS (Sergeyev et al. 2005).

2.3.4 Contradictions in the CMS literature

Much of the early literature about CMS comes from work carried out in the laboratory in which the procedure was developed. This raised the problem of the reliability and reproducibility of CMS elsewhere. In recent years CMS has been adopted in several laboratories (Grippo *et al.* 2003; Konkle *et al.* 2003; Kang *et al.* 2005). Many of them have established the procedure successfully, others have raised several points of criticism of the CMS model (Nielsen *et al.* 2000). The most contradictory issue in the CMS literature is related to the inconsistent occurrence of the anhedonic effect (see e.g. Psychopharmacology, 134, 1997). CMS is argued to cause a generalized decrease in sensitivity to rewards. Some researchers claimed that the decrease in reward responsiveness, i.e. changes in intake of sweet solutions, may result from artifacts related to loss of body weight (Matthews et al. 1995; Forbes et al. 1996). Others found that the decrease in sucrose intake in CMS animals is much larger than the decrease in body weight (D'Aquila *et al.* 1997). Changes in body weight are not consistently observed after CMS.

Another critique of the CMS model is based on the claim that elements in the CMS protocol (e.g. the food and water deprivation) may be sufficient to produce a decreased consumption of palatable solutions (Hatcher et al. 1997). However, experiments in which CMS and Control animals were both exposed to food and water deprivation showed that decrease in sucrose intake only occurs in CMS animals (Willner *et al.* 1992; Valverde *et al.* 1997). Some laboratories found that CMS-induced decrease in sucrose/saccharin intake is accompanied by decreases in preference for the solutions (Willner *et al.* 1987; Ayensu *et al.* 1995; D'Aquila *et al.* 1997). Others have found decrease in sucrose intake while sucrose preference remained unaltered (Matthews *et al.* 1995; Forbes *et al.* 1996). Decrease in sucrose preference may even be detected in the absence of a decrease in sucrose intake (De Vry and Schreiber 1997). Another measure of reward behavior is ICSS. Only one laboratory has reported consistent ICSS changes after CMS (Moreau *et al.* 1992; Moreau *et al.* 1995). Another laboratory has emphasized that replication of CMS-induced decrease in ICSS can be as problematic as inducing decrease in sucrose intake (Nielsen *et al.* 2000).

Other physiological alterations induced by the CMS protocol have proved to be inconsistent. For instance, some studies have demonstrated an elevation of basal corticosterone levels in rats exposed to CMS (Ayensu *et al.* 1995; Bielajew *et al.*

2002), whereas others have found biphasic responses (Silberman et al. 2002), no change (Azpiroz et al. 1999), and even decrease in corticosterone levels following CMS (Murison 2001).

To date there is no obvious explanation for the apparent difficulties in establishing the CMS model across laboratories (see comments to review (Willner 1997). The picture is made complex by the numerous ways CMS experiments are performed. The type and duration of stressors applied, the time point and frequency of the stressors administration are seldom reported, leaving the debate open.
Physiological measures as temperature and heart rate are found to fluctuate markedly depending on the time and stressor applied (Nielsen 2000). Hence, the different CMS regimes may not possess equal strength and the lack of expected behavioral outcome may be due to an insufficient stress level. To avoid interference with the acute CMS stressors, all rats should be left without any treatment for at least 12-24 h before measuring any CMS effect. This is not often specified in the literature.
Finally, experimental outcome may depend on the animal strain used. The reproducibility of the CMS effects seems to be sensitive to genetic variability and to environmental factors usually not accounted for. Strain-related difference in response to CMS is present (Pucilowski *et al.* 1993; Overstreet *et al.* 1997; Nielsen *et al.* 2000). 1997). Exposing Flinders Sensitive Line, a genetic animal model of depression selectively bred for its increased response to anticholinesterase agents, to CMS, leads to a greater reduction of saccharine intake compared to the control strain Flinders Resistant Line (Overstreet *et al.* 1997).

3 AIMS OF THE STUDY

We wanted to establish an animal model of depression in our laboratory and further study some pathophysiological and neurobiological aspects in the model.
In particular, we intended to investigate behavior (e.g. sleep, locomotor activity, etc.) and neuromodulation linked to depression. CMS has a high degree of validity that makes it suitable for this purpose. Also, CMS was especially chosen because it mirrors daily hassles of human life. However, as previously discussed, it is somehow problematic to reproduce this model (e.g. to produce anhedonic animals that consume less sucrose than controls). Many attempts to replicate the original model have failed

in a variety of laboratories. This made it necessary to confirm the effect of the CMS protocol before proceeding to additional evaluations. The project developed according to the following steps:

1) Establishment of the CMS model in our laboratory.
    a) administration of stressors
    b) assessment of anhedonia (sucrose/saccharine intake)
    c) monitoring body weight
2) Evaluation of pathophysiological aspects of depression in the CMS model
    a) plasma corticosterone
    b) sleep alterations
    c) sexual behavior
    d) locomotor activity and emotionality
3) Evaluation of neurobiological aspects of depression in the CMS model
    a) BDNF, p-CREB and total CREB expression in the hippocampus
    b) extracellular 5-HT level in the hippocampus and sleep dependent changes.
4) Correlation between anhedonic behaviour (sucrose intake) and pathophysiological/neurobiological measures.

4 MATERIALS AND METHODS

An overview of the methods is presented here. For more details, see each individual article.

4.1 Ethical Approval

The experiments described in this thesis have been approved by the Norwegian Animal Research Authority and registered by the Authority. The experiment has thus been conducted in accordance with the laws and regulations controlling experiments in live animals in Norway, i.e. The Animal Protection Act of December 20th 1974, No 73, Chapter VI sections 20-22 and the Animal Protection Ordinance concerning Biological Experiments in Animals of January 15th 1996. 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.

4.2 Animals

Sprague-Dawley (Mol:SPD) strain, male and females, were purchased from Møllegaard, Copenhagen, Denmark and used in this thesis. The animals weighed 230-260 grams on delivery. They had free access to tap water and food (Rodent low protein diet, B&K Universal, Norway) unless otherwise stated. Housing conditions were controlled, temperature was maintained at 22 ± 1^{o}C with 52 ± 2 % humidity. The rats were kept on a controlled 12:12 light/dark cycle with increasing lights from 0600h and fully lights on at 0700h (Paper I) and a reversed L/D cycle in Paper II, III and IV. To minimize stress, the animals were allowed to remain in their transport cage for five days before they were housed individually. A period of at least one week for adaptation to the laboratory facilities was allowed. All handling were performed by the same person throughout the studies.

4.3 CMS procedure

In each experiment, the animals were divided into two matched groups based on their sucrose consumption in preliminary tests (1% sucrose solution). One group was given ordinary daily care (Control rats) and the other group was exposed to chronic mild stress. The two groups of rats were housed separately in different rooms during the duration of the stress procedure.

The CMS protocol with clock time and duration of the procedures is shown in Paper I and PaperII, Figure 1, bottom. Most of the stressors were adapted from the procedure described by Willner and collaborators (Willner *et al.* 1987) and some stressors were included from Moreau and collaborators (e.g. empty water bottle, restricted food) (Moreau *et al.* 1992). Each week included 2 h of paired caging, 3 h of tilted cage (45 degrees), 18 h of food deprivation immediately followed by 1 h of restricted access to food (5 micropellets), 2 x 18 h of water deprivation immediately followed by 1 h exposure to an empty bottle, 21 h of wet cage (200 ml water in 100 g sawdust bedding), and 36 h of continuous light. Stressors were presented both during

the rats' active (dark) period and during the inactive (light) period. The same stressors were used inn all experiments.

4.4 Sucrose intake, saccharine intake and body weight

Sucrose intake (1% sucrose solution) was measured once a week, during a one-hour window after four hours of food and water deprivation. In Paper II also saccharine intake (0.1% saccharine solution), on a separate day, was measured. The consumption was measured by comparing bottle weight before and after the one-hour window, and expressed in relation to the animal's body weight (ml/kg). Baseline was measured five days before the start of CMS. The food and water deprivation period preceding sucrose/saccharine intake measurement may be considered as a further stress applied on top of the CMS protocol. However, control rats were also exposed to the food and water deprivation, as a part of the sucrose test.

4.5 Endocrinological measure

To avoid interference with sleep recording and possible effects of CMS stressors, the blood samples for analysis of corticosterone were taken the day following sleep recording and when no stressor had been applied to the rats for two days. Blood samples were taken from the rats between 0900 h and 1200 h. To avoid activation of the HPA axis, the time used from moving the animals from home-cages to completion of blood collection was less than 3 min. The animals were transported to the sampling laboratory, removed from their home cages and placed in a sealed anesthetic chamber. Anesthesia was induced with Isofluran. Following muscle relaxation, the rat was removed from the chamber and placed in a dorsal position and a controlled amount of anesthetic gas was given through a mask covering mouth and nose. An incision was made on the right side of the throat, exposing the jugular vein. 1 ml blood sample was collected, centrifuged and plasma drawn off and frozen at -20^0C until further analysis. Serum corticosterone (ng/ml) was measured by radioimmunoassay using a commercially available reagent kit (Count-A-Coat, Diagnostic Products Corporation, Ca).

4.6 Behavioral measures

4.6.1 Sleep recording. Procedures and analysis

Surgical procedure: Animals were implanted under anesthesia (a mixture of fentanyl 0.05 mg/ml, fluanizone 2.5 mg/ml and midazolam 1.25 mg/ml, Hypnorm Janssen; Dormicum, Roche) with the standard set of electrodes for polygraphic recording (see: Ursin and Larsen 1983) and Paper I and III/IV. In brief, electroencephalogram (EEG) electrodes were implanted for bilateral fronto-frontal (FF) and fronto-parietal (FP) recording and electromyogram (EMG) electrodes were inserted in the neck musculature. The frontal electrodes were placed 2 mm anterior to bregma and 2 mm lateral to midline, the parietal electrodes were placed 2 mm anterior to lambda and 2 mm lateral to the midline. All electrodes were connected to a socket which was anchored to the skull with dental cement (Paladur, Kulzer & Co., Germany). In Paper III/IV, the rats were in addition implanted with an intracerebral guide cannula that allows easy insertion of the microdialysis probe. Bregma coordinates for hippocampus were AP = -5.8, ML = -5.0, DV = -8.0. The animals received an analgesic twice a day for three days following the surgery (0.15 ml Temgesic s.c.) and were allowed to recover for 2 weeks before adaptation to the recording condition.

Polygraphic recording. Following surgery and recovery, each animal was habituated to the recording chamber for 3 days, at least 6 h per day, preceding recording days. The animals remained in their home cages which were placed into the electrical shielded and sound attenuated recording chambers and had free access to food and water. Free movement was permitted by a flexible recording cable and electrically swivel attached to a movable arm placed outside and above the chamber. The animals were recorded for 8 h, beginning one hour into the light period. The rats were not exposed to any stressor for at least 12 h before the sleep recording.

Data analysis: EEG and EMG were recorded and digitized at a sample frequency of 100 and 200 Hz, respectively. For visual display and scoring, the filtering of the EEG signals was set at 35 Hz for the low-pass filter and at 3 and 5 Hz for the high-pass filter of FF and FP, respectively. The EMG signal was filtered with a high-pass filter at 5 Hz. All signals were filtered with 3 dB/octave filter.

Sleep and waking were scored manually in 10 sec epochs and classified in 4 stages (W, SWS-1, SWS-2, REM sleep) according to the criteria given by Ursin and Larsen (Ursin and Larsen 1983) and described in detail in Paper I. The number of sleep

episodes, duration and latency for each stage were computed using the sleep-dedicated software Somnologica. Latencies to SWS-2 and REM sleep were scored from sleep onset to the first epoch of that sleep stage. If rats were asleep when sleep recording started, sleep onset was set at 0800 h (Paper I) and 2000 h (Paper IV). In Paper IV the animals were kept awake until start of the recording to measure the latency to SWS-2 and REM sleep.

4.6.2 Sexual activity

The female rats were ovariectomized and brought into oestrus before the mating test by injections of oestradiol benzoate (200 μg/rat in oil, s.c.) and progesterone (0.5 mg/rat in oil, s.c.), 48 and 6 h before the mating test, respectively. Prior to the experimental day, each male underwent 3 preliminary mating tests. This allowed us to identify any apparent noncopulator. The males were included in the experiment if they had a total of three ejaculations. A female in oestrus was introduced into the home cage of the male and the copulatory behavior were recorded for 30 min online on an event recorder and taped by a videocamera. The latency and the frequency of the following measures of copulatory behavior were scored: mounting, intromission and ejaculation. Mating tests were carried out 2 h into the dark phase in a room lit by a dim red light. The rats were not exposed to any stressor for at least 12 h before the test.

4.6.3 Open field test

The open field consists of black walls (20 cm) and a black base (100 cm x 100 cm) divided into 25 (5 x 5) identical sectors (20 cm x 20 cm) by white stripes. The squares were subdivided into peripheral and central sectors. The central sector consisted of the 9 central squares (3 x 3) and the peripheral sector contained the squares close to the wall. The rats were placed in the central sector and their activity recorded for 6 minutes by a videocamera and taped for further analysis. The total locomotor activity is expressed as total activity, while activity in the central or peripheral sectors give a measure of exploratory behavior or harm avoidance respectively. The first minute activity and the latency to move to the peripheral sector are used as a measure of emotionality.

Experiments were performed 2 h into the dark phase and no stressor was applied to the animals for at least 12 h before the test. The room was lit by a red light.

The rats were left alone in the room during the test and the base was thoroughly cleaned between each test.

4.7 Analysis of BDNF, p-CREB and total CREB

The relevant brain hemisphere was rinsed with oxygenated ice-cold artificial cerebral spinal fluid (aCSF). The dentate gyrus and CA region were rapidly dissected on an ice-cold glass dish, aliquoted into Eppendorf tubes, and stored at –80^{o}C until analysis.

Primary antibodies used were BDNF (N-20) Sc-546 rabbit polyclonal IgG (1:2000) (Santa Cruz), total CREB rabbit polyclonal (1:2000) (Upstate) and phospho-CREB at Ser 133 rabbit polyclonal (1:2000) (Santa Cruz). Secondary antibody used was goat anti-rabbit IgG (1:5000) (Calbiochem).

Tissues were hand-homogenized with 15 strokes in 300 µl of Dynal lysis/binding buffer. Protein levels in homogenate samples were determined using the Lowry method. Equal amounts of protein were loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Phosphorylated CREB and total CREB on 10% gels and BDNF on 12% gel and run overnight at constant 10 mA. Separated proteins were transferred onto a nitrocellulose membrane (Hybond-C Amersham) at constant voltage of 30 V overnight or 100 V for 1 h. Membranes were blocked on a gyro-rocker for 1 h at room temperature. Blocking buffer consisted of TBST (Tris-buffered saline/0.1% Tween-20) and 5% albumin from bovine serum (BSA). The primary antibodies were dissolved in blocking buffer containing 3% BSA and the blots for BDNF and total CREB were incubated for 2 h at room temperature and p-CREB was incubated at 4°C overnight with constant shaking. Following three washes with TBST, blots were incubated for 1 h in horseradish peroxidase-conjugated secondary antibody dissolved in TBST. The blots were washed three times with TBST and proteins were visualized using enhanced chemiluminescence (ECL Western Blotting Analysis System, Amersham pharmacia biotech, Norway). Autoradiographs were scanned on a densitometer and quantitated using Phoretics ID plus software.

4.8 Analysis of 5-HT

4.8.1 Microdialysis

The technique of brain microdialysis coupled with electrochemical detection is a way to monitor the level of monoamines in freely moving animals. The method samples the extracellular fluid and allows detection of neurotransmitters and other substances from the brain. The *in vivo* recovery of a substance depends on its concentration in the surrounding tissue. Chemicals move across the microdialysis membrane to and from the extracellular fluid following diffusion along a logarithmic concentration gradient. The probe was inserted at least 16 h before the experiment start. A recovery period after insertion is necessary e.g. for clearance of transmitters leaked from terminals damaged by probe insertion. The microdialysis probe (CMA 12) had a diameter of 500 μm, a membrane length of 4 mm and a cutoff of the membrane (polycarbonate/polyether copolymer) on 20 000 Dalton MW. Relative recovery rate of the probe was in the range of 45 – 50%. Starting in the beginning of the animal light period, the probe was perfused with aCSF (147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$ and pH 7.2) and the flow rate maintained at 1.2 μl/min by a microdialysis pump. In our experience, the dialysate values are constant after 1 h after start of the perfusion. The animals were left undisturbed during the experiment and samples from each animal were manually collected during polygraphically defined behavioral states. An average of 4 – 6 samples was collected from W and SWS and 1-2 from REM sleep. The short length of each REM sleep episode made it necessary to add two or more REM sleep samples in order to obtain 7 μl of dialysate. The samples were frozen immediately at –80^{o}C for later analysis.

4.8.2 Separation and detection of 5-HT

The samples (7 μl) were analysed for 5-HT by using a high performance liquid chromatography (HPLC) system. The separation and detection was achieved using a microbore column (3 μm, ODS, 100 x 1 mm) coupled to an electrochemical detector (UniJet LC-4C amperometric detector, BAS). The potential applied to the glassy carbon electrode was 550 mV with respect to the reference electrode. The sensitivity was set to 1 nA full scale. For the contents of the mobile phase, see Paper IV. Chromatographic data were recorded and the 5-HT peak was identified by comparison with 5-HT standards and by using the technique of ‘spiking’.

Concentrations were determined using an external standard calibration curve method. The detection limit for 5-HT in this study was approximately 0.1 fmol.

4.8.3 Histology

At the end of the experiment, rats were anesthetized with Isofluran gas and decapitated. Brains were rapidly removed from the scull and the hemisphere containing the site of the microdialysis probe was stored in 15% sucrose/0.1 M phosphate buffer until equilibration. The location of the probe was determined according to the atlas of Paxinos and Watson (1998) in 40 μm thick serial coronal slices cut on a freezing microtome and stained with Giemsa.

4.9 Statistics

In the CMS protocol, the important variable is represented by the individual animal's response to the treatment. There is often considerable variation between animals in regard to consumption of palatable solutions, behavior and their response to the CMS procedure. Thus, in the analysis of CMS data it is important to make between- and within group comparisons.

Sucrose intake, saccharine intake and body weight: Both the comparison between group and the effect of CMS or control treatment (within groups) was performed with analysis of variance (ANOVA) for repeated measures (over days). Any difference between baseline consumption and day was assessed by multiple comparisons performed by least significant deviation (LSD) post hoc test.

Corticosterone: Levels of corticosterone following CMS or control condition were analysed with Student's *t*-test.

Sleep and waking stages: Sleep data between groups were analyzed using a 4-way ANOVA for repeated measures ('group' as independent factor and 'recording day', 'sleep stage' and '2h period' as repeated measures). The effect of the CMS or control condition (within-group) on recording day was analysed with 3-way ANOVA for repeated measures ('recording day', 'stage' and '2h period') and a 2-way ANOVA for further analyses of sleep and waking stages ('stage' and '2h period'). Differences between 2h periods were evaluated by Student's *t*-test.

Difference in latency to sleep stages between recording days was assessed by Student's *t*-test.

Fragmentation and duration of sleep episodes were analysed by 2-way ANOVA ('recording day' and 'number of episodes'). Difference between recording days was evaluated by Student's *t*-test.

Sexual activity and open field data: The differences between the groups of latencies and frequencies of sexual behavior ('mount', 'intromission', 'ejaculation') and the activities measured in the open field test (center, peripheral, total and first minute activity) were analyzed using the non-parametric Friedman ANOVA.

BDNF, p-CREB and total CREB: Differences between groups were analyzed with Student's *t*-test, assuming equal variance.

Correlation analysis: Pearson's correlation analyses were performed on pooled data, from both Control and CMS rats.

Extracellular levels of 5-HT: The 5-HT data (fmol) were not normal distributed (Shapiro-Wilk test). Therefore, 5-HT in SWS and REM sleep were converted to percent compared to their own W value. Group effect of normalized data was assessed by a two-way ANOVA ('group' x 'stage'). Analysis of stage effects within the groups were performed with a one-way ANOVA. LSD test was used for *post hoc* comparisons.

All statistical data analyses were performed using Statistica 5.0. One-tailed probability values were used in cases where there was a strong experimental hypothesis (e.g. increased REM sleep, reduced sexual activity). Otherwise, significance was accepted at $p < 0.05$, two-tailed.

5 SUMMARY OF THE RESULTS

The experiments were performed in our laboratory during a 3 year period and the results are presented in full in Paper I, II, III and IV.

5.1 Establishment of the CMS model

In addition to the CMS protocol described by Willner (1987) we added an extra stressor; i.e. an empty water bottle following water deprivation and food

restriction after total food deprivation (Paper I, II, III). This may have had a reinforcing effect on the CMS protocol.

The consumption of sweet solutions varied in our CMS experiments. Paper I confirmed that the CMS procedure in rats decreases sucrose intake per unit body weight. This reduction of sucrose intake was maximal at 2 weeks and returned to baseline level after four weeks. Also, in Paper II the sucrose consumption was reduced, however the max intake response occurred after 2 weeks and lasted throughout the CMS protocol. In another experiment (Paper III), CMS rats did not show a decreased consumption compared to baseline level, however, their sucrose intake was lower than Controls throughout the CMS protocol.

In Paper II the effect of CMS was tested on both sucrose and saccharine solutions in the same animals. The consumption of the two sweet liquids was not equally affected. CMS reduced intake of sucrose but had no effect on saccharin consumption.

Our experimental rats did not lose any weight (Paper I, II, III), but gained weight as rapidly as the Controls during the CMS protocol.

5.2 Pathophysiological consequences of CMS

Rats exposed to CMS did not show changes in plasma corticosterone level compared to Control rats (Paper I).

Sleep/wake changes after CMS mimic to some extent sleep alterations found in depressed patients (Paper I, replicated in Paper IV). CMS produced selective changes in both the structure and the continuity of the sleep, by increasing the time rats spent in REM sleep and SWS-1, and producing a less stable sleep/wake pattern. The sleep fragmentation was due to increased number of REM sleep and SWS-1 episodes. Also, in Paper I, the duration of SWS-2 and waking episodes were decreased, duration of SWS-1 episodes increased and the number of waking episodes were higher. However, in none of these studies did CMS change the latency to REM sleep or SWS-2.

Paper II shows that CMS reduced male sexual activity. CMS rats had longer latencies to intromission and ejaculation, and achieved fewer ejaculations compared to Controls.

The open field test showed an increase in spontaneous activity following CMS, especially in the first minute of the test (Paper II). Also, CMS rats showed a trend of higher activity in the central sector of the open field.

5.3 Neurobiological consequences of CMS

CMS induced specific inhibition of BDNF expression and CREB activation in the dentate gyrus (-25% and -30%, respectively). These measures were not significantly changed in the CA. The expression of total CREB and the control protein β-actin were unaffected by CMS (Paper III).

The extracellular waking level of hippocampal 5-HT was not affected after CMS. However, a trend towards an increase was present in SWS and REM sleep (Paper IV).

5.4 Correlation of anhedonic behavior (sucrose intake) and pathophysiological/neurobiological measures

If a reduced sucrose intake reflects anhedonia in rats, its value should correlate to other alterations observed after CMS. In Paper I, a correlation between sucrose consumption and some sleep alterations was found. The animals that consumed less sucrose spent more time in REM sleep, had more REM sleep episodes and a shorter duration of SWS-2 episodes. Paper III shows a positive correlation between sucrose intake and p-CREB expression in dentate gyrus (but not in CA). In Paper IV, we found that the rats consuming less sucrose were those having the highest levels of hippocampal 5-HT in SWS and REM sleep compared to the waking state.
A correlation between sucrose intake and plasma corticosterone was not present (Paper I).

6 GENERAL DISCUSSION

The CMS model was successfully established in our laboratory. CMS rats showed anhedonic behaviour measured as sucrose intake. In addition, several significant behavioral and neurobiological changes were observed as a consequence of CMS.

6.1 Establishment of CMS as an animal model of depression

The central focus and the theoretical rationale of the CMS model is based on the induction of anhedonia, defined either as 'a loss of responsiveness to pleasant events' or 'as a generalized decrease in sensitivity to rewards'. A reduced consumption of palatable solutions of sucrose or saccharin is the hedonic measure that has been most widely used (Willner *et al.* 1987; Ayensu *et al.* 1995; Papp *et al.* 1996; Marona-Lewicka and Nichols 1997). The CMS model has been criticized because of the inconsistent outcome of stress-induced anhedonia (Matthews *et al.* 1995; Forbes *et al.* 1996; Harris *et al.* 1997; Hatcher *et al.* 1997; Nielsen *et al.* 2000). In our studies, CMS rats consumed less sucrose compared to the Control group. However, sucrose consumption varied between animals and across experiments. In paper I and II a significant decrease was found. However, max intake response occured at different times during the CMS protocol. In Paper I the largest effect was observed after 2 weeks of CMS, and then the effect attenuated. In Paper II the max intake response occurred after 2 weeks and lasted throughout the CMS protocol. In Paper III the CMS rats did not decrease the sucrose intake compared to their baseline, however, their consumption was lower than Controls' throughout the protocol. We also observed variations in sucrose consumption in Control animals. In paper I and II the variations parallel CMS rats' decrease of sucrose intake, in particular the decrease observed during weeks 2-4 (Paper II). In paper III there was an increase in the sucrose consumption in Control rats. There is no obvious explanation to such spontaneous fluctuations. These variations have also been observed by others (Katz 1982; Willner *et al.* 1987; Matthews *et al.* 1995; Phillips and Barr 1997; Kioukia *et al.* 2000; Konkle *et al.* 2003).

We also tested sensitivity to consumption of different sweet solutions. To our knowledge, Paper II is the first study testing both palatable solutions in the same CMS experiment in the same animals. The results show that sucrose intake and saccharine intake are differentially affected by CMS, as the two measures do not correspond in the same animal. Saccharine intake was not affected by CMS. Harris and collaborators (Harris *et al.* 1997) showed that a decrease in saccharin intake occurs if the period of water deprivation preceding the test is longer than 24 h. Our protocol for the consumption of sweet solutions included 4 h of water deprivation. The choice of a 4 h limit for the water deprivation period was motivated by the fact that dehydration produces metabolic effects unrelated to the CMS protocol. Variability between the protocols used for testing intake of palatable solutions in different laboratories may explain some of the controversial findings observed in the literature (Ayensu *et al.* 1995; D'Aquila *et al.* 1997).

It has been suggested that reduction of body weight following CMS contributes to a decrease in sucrose intake (Matthews *et al.* 1995). However, all our experiments show that bodyweight is not associated with consumption of sucrose. CMS rats consume less sucrose but gain weight in the same manner as the Control group (Paper I, II and III).

Overall, our results show that the CMS rats consumed less sucrose compared to the Control group. In addition sucrose intake correlated with many depression-related variables strengthening the role of CMS as a model of animal depression. The CMS model is attractive because it displays multifaceted stress-induced alterations that mirror the complex nature of depression made up of a variety of emotional, behavioral and cognitive elements. However, the model also presents some limitations. CMS studies are expensive, work intensive, space demanding, long lasting, and there are difficulties in establishing the model in new laboratories. In our experience, care should be taken to control any environmental stressor that may affect a CMS experiment. Pre- and postnatal events beyond the experimenters' control may affect animals' vulnerability to stress in later life.

6.2 The CMS effect on pathophysiology

We could not find signs of increased activity in the HPA axis as measured by plasma corticosterone (Paper I). In other CMS studies plasma corticosterone was found to be increased, not changed or decreased (Ayensu *et al.* 1995; Azpiroz *et al.* 1999; Murison 2001; Bielajew *et al.* 2002; Silberman *et al.* 2002). In depressed patients, several studies show a hyperactive HPA axis (for review see (Plotsky et al. 1998). In Paper I, a recovery of 'reward behavior' was observed at the end of the protocol. Since plasma corticosterone was measured at the end of the CMS protocol and since no stressors had been applied to the rats for two days, it is possible that the corticosterone response was attenuated.

Specific sleep alterations are often used as a physiological marker for depression. We found changes in both the structure and the continuity of sleep after CMS. Most of the changes (especially increased REM sleep, fragmented sleep, reduced deep sleep) parallel changes observed in human depression. In human studies, the increase in REM sleep is usually seen in the first half of the night (Reynolds and Kupfer 1987). In our CMS rats this effect differed. An increase was observed in the first 2h period (Paper IV) but also in the last half of the night (Paper I). Other CMS studies show the occurrence of the REM sleep increase in the last half of the night (Moreau *et al.* 1995; Cheeta *et al.* 1997). Our CMS rats also paralleled the less stable sleep-wake pattern often observed in depressed patients (Kupfer and Reynolds 1992). The increased sleep fragmentation was due to an increased number of REM sleep and SWS-1 episodes (Paper I and IV) and waking (Paper I). One might argue that some of these findings may reflect a rebound due to sleep loss during the actual stress exposure. In rats, sustained stress disturb the distribution of sleep over the 24 h cycle by reducing the amount of sleep in the light phase (deactive phase) compensated by an increase in sleep during the dark phase (Kant *et al.* 1995). In humans, repeated partial sleep deprivation results in increase of the deep SWS (Webb and Agnew 1965; Dement and Greenberg 1966; Brunner *et al.* 1990) and a delayed and prolonged REM sleep rebound (Brunner *et al.* 1993). CMS animals did spend more time in REM sleep, but also showed fragmented sleep and reduced SWS-2. Since the rats were not exposed to any stressor at least 24 h before the sleep recording it is most likely that the sleep changes were the result of the CMS procedure.

CMS changed sexual behavior in rats. Sexual behavior has been divided in motivational and executive components. CMS rats showed longer latencies to intromission and ejaculation and achieved fewer ejaculations. However, a decrease in the intromission frequency was not found. This might indicate that CMS seems not to induce a decreased capacity of the animals to achieve penile erection. It is likely that the finding of an increased latency to interact is responsible for the abolishment of ejaculation. The latency to intromission is linked to the motivational component. An alteration in the sexual behavior located in the motivational component during the 30-min test was an expected consequence of the CMS protocol, reflecting a diminished motivation manifested in sexual function commonly found in human depression (Shabsigh *et al.* 2001).

When placing the rats in a novel environment (open field test), the CMS rats showed a higher locomotor activity compared to Controls, especially in the first minute of the test (Paper II). Low activity in an open field test has been used as an index of high emotionality in rats (Walsh and Cummins 1976; Royce 1977; Katz et al. 1981). However, Denenberg (Denenberg 1969) interpret high activity in the first exposure and low activity in the following exposures to a novel environment as indicative of increased emotionality. This view is supported by Piljman and collaborators (Pijlman et al. 2003) showing a reduction in locomotor activity after physical stress, whereas emotionally stressed rats show an increased activity. We only exposed the rats to the open field once and interpret our findings as indicative that the CMS model increases the emotionality in rats. In addition, our CMS rats showed a tendency to increased activity in the central sector when they were placed in an open field apparatus. The ability of an animal to adjust its behavior in anticipation to changing environmental hassles is thought to be an adaptive response. It is reported that this response is facilitated by aversive responding induced by previous adverse experiences (Korte 2001). Hence, this suggests that our CMS rats did not adapt to the chronic stress protocol (Paper II).

6.3 Neurobiological alterations after CMS

CMS induced a specific inhibition of the BDNF – CREB system in the dentate gyrus. We did not observe significant changes of BDNF or p-CREB in the CA region which is described in more severe stress protocols (Smith *et al.* 1995; Russo-Neustadt *et al.* 2001). This could indicate that the dentate gyrus is an area more vulnerable to a prolonged mild stress exposure. Such sensitivity may be mediated by different stress-sensitive brain circuitries, e.g. the involvement of limbic and neocortical forebrain structures. This is in line with the concept of differential circuitry being involved in the brain processing of psychological (e.g. chronic stress, predator exposure) versus physical stress (e.g. cold, exposure to ether) (Herman and Cullinan 1997). The decrease in the expression of BDNF protein and phosphorylated CREB in CMS animals are more likely to be of psychological nature since they were measured 2 days after the final stress session. This delay avoids the confounding effects related to an acute response to stress.

A decreased expression of BDNF appears to be associated with depression-like symptoms in animals and with depression symptoms in humans. A decreased serum BDNF level has been reported in human depression (Karege *et al.* 2002) and has been found to be an indicator of vulnerability to develop depression (Lang *et al.* 2004). Several other animal models of depression have shown a reduced expression of BDNF in different brain regions (e.g. maternal deprivation, footshocks, cold swim, restraint stress, social defeat, prenatal stress, learned helplessness (Smith *et al.* 1995; Russo-Neustadt *et al.* 2001; Rasmusson *et al.* 2002; Roceri *et al.* 2002; Fumagalli *et al.* 2004; Itoh *et al.* 2004; Pizarro *et al.* 2004)). Only a few animal models of depression do not show a decreased expression of brain BDNF (e.g. some genetic animal models of depression (Angelucci et al. 2000; Vollmayr et al. 2001)). BDNF may play a role in the mechanisms underlying antidepressant action. In patients treated with antidepressant medication at the time of death, BDNF expression was increased in the hippocampus compared to untreated patients (Chen et al. 2001). In rats, chronic (but not acute) treatment with antidepressants and electroconvulsive shock treatment increase BDNF mRNA in the hippocampus (Nibuya *et al.* 1995; Russo-Neustadt *et al.* 2000).

The dentate gyrus has emerged as a critical site in the pathogenesis of depression and the action of antidepressant drugs. Recent work suggests that the

dentate gyrus functions in fine spatiotemporal separation of novel and complex cues (Kesner et al., 2004;Lee et al., 2005), suggesting that the dentate gyrus may disambiguate stimuli to allow sparse encoding of information (Gould and Cameron 1996). A dysfunction in dentate gyrus fits with the emotional withdrawal, social isolation, and impaired memory in depressed patients. The dentate gyrus is one of few brain regions where robust neurogenesis, the birth of new neurons, continues into adulthood (Altman and Das 1965; Gould and Cameron 1996). Regulation of granule cell survival and neurogenesis is promoted by BDNF. In addition, BDNF is a major regulator of activity-dependent synaptic plasticity represented by long-term potentiation (LTP) in the dentate gyrus (Bramham and Messaoudi 2005). This requires new gene expression and typically involve activation (phosphorylation) of the transcription factor CREB (Tao *et al.* 1998; Grewal *et al.* 1999). CREB expression and function are up-regulated by chronic antidepressant treatment in rodents and humans (Nibuya *et al.* 1996; Dowlatshahi *et al.* 1998; Thome *et al.* 2000). Induced over-expression of CREB in the dentate gyrus produces an antidepressant-like response in learned helplessness rats and in the forced swim test (Chen et al. 2001). This is in line with our results showing a marked reduction of p-CREB in the dentate gyrus in CMS rats. It is likely that the BDNF-CREB system is involved in development of depressive episodes and that a downregulation of the system during CMS impairs the information processing and storage capacity of the dentate gyrus.

The individual expression of p-CREB in dentate gyrus was positively correlated with the animals' sucrose intake. The rats consuming less sucrose solution showed the lowest expression of p-CREB in the dentate gyrus. Hence, p-CREB appears to be a sensitive measure of the pathophysiological changes associated with depression-like symptoms. A hippocampal-ventral tegmental area (VTA) loop has been proposed to be involved in memory formation. According to this hypothesis, novelty detection in the hippocampus leads to activation of the VTA (a key area of the dopaminergic reward system), release of dopamine, and facilitation of LTP in the hippocampus (Lisman and Grace, 2005). While speculative, it is conceivable that decreases in reward activation and dopamine release contribute to impaired CREB activation in the CMS model. The present study supports the hypothesis that the levels of hippocampal BDNF may be decreased in depression. However, we did not find a correlation between sucrose intake and BDNF. This might indicate that changes in

BDNF protein levels and CREB activation in the dentate gyrus are not necessarily inter-dependent in relation to depression.

According to the monoamine hypothesis of depression (Maes M 1995) and a dysfunctional serotonin system of depressed patients (Mann et al. 1990, Sarrias et al. 1987), we expected to find a lower level of extracellular 5-HT in the CMS model of depression. However, CMS did not decrease the hippocampal 5-HT compared to Control condition (Paper IV). If anything, a change toward an increase seemed to appear during sleep. This possibility was supported by a positiv correlation between the rats' anhedonic behavior and level of 5-HT in sleep. The rats that consumed less sucrose were those having the highest levels of hippocampal 5-HT in SWS and REM sleep relative to their waking state.

Stressful events have been reported to cause various neurochemical changes, which have similarities with those associated with depression (Anisman and Zacharko 1990). Studies on the serotonergic activity in hippocampus after CMS have shown different results. In a microdialysis study, Kang et al. reported a reduction of the extracellular 5-HT (>20%) compared to Control rats (Kang *et al.* 2005), while in homogenized brain tissue an increase (Bekris et al. 2005), decrease (Li et al. 2003) and no change (Haidkind *et al.* 2003) have been described. However, there are no indications that the serotonergic neurons of the dorsale raphe nucleus change their discharge rate during stress (Jacobs and Azmita 1992, Maudhuit et al. 1997). Instead, stress has been shown to alter 5-HT neurotransmission in regions to which the DRN projects, including the hippocampus (Kirby and Lucki 1998, Linthorst et al. 1996, Torres et al. 2002, Rueter and Jacobs 1996).

Serotonergic function is dependent on the availability of neurotransmitter in the synaptic cleft and on receptor binding activity and sensitivity (Struder and Weicker 2001). There is a dense concentration of 5-HT_{1a} receptors in the hippocampus (Kia et al. 1996). In contrast to other models involving repeated stress (Watanabe *et al.* 1993), CMS has been found to increase the binding to 5-HT_{1A} receptors in the hippocampus, and a further increase by imipramine treatment has been found (Papp *et al.* 1994). Also, CMS desensitize the inhibitory 5-HT_{1A} autoreceptors in DRN (Froger et al. 2004) suggesting that 5-HT output may increase as a result. A 5-HT_{1A} agonist, believed to be acting postsynaptically, has been shown to normalize the CMS induced decreased sucrose intake in rats (Munoz and Papp

1999). Hence, it is possible that the effect of CMS on serotonergic activity largely derives from a direct action on 5-HT_{1A} receptors.

Due to the low number of animals in Paper IV however, particularly in the Control group, the findings would have to be replicated. Nevertheless, it was notable that the rats consuming less sucrose showed highest levels of 5-HT in SWS and REM sleep. The reason for this effect is not clear. CMS rats showed typically REM sleep alterations in depression suggesting that more dishinibition of REM-promoting neurons may occur. REM promoting neurons are under the inhibitory influence of 5-HT (Leonard and Llinas 1994) and REM sleep occurs when serotonergic output is lowest (Lydic et al. 1987). We measured 5-HT in the hippocampus, a representative serotonergic projection area where a state dependent level of 5-HT is reported (W>SWS>REM sleep) and parallels the state-dependent changes present in other serotonergic projection areas (including REM promoting regions of the pons) (for review (Portas et al. 2000)). In this view, we suggest that a dissociation between the level of hippocampal 5-HT and the occurrence of REM sleep may take place after CMS.

Serotonergic activity is known to be low during SWS (Lydic *et al.* 1987). This was not seen in the CMS group. Hence, it appears that CMS, produces a paradoxical effect on serotonin in SWS and REM sleep. Neurochemically, the sleep/wake cycle is associated with interactions between aminergic and cholinergic neurotransmission (Steriade and McCarley 1990), the regulation of neuropeptides (Ehlers and Kupfer 1987) and other systems. A decreased GABAergic function have been reported in animal models of depression (Sherman and Petty 1982; Jancsar and Leonard 1984; Borsini et al. 1986) and in humans affected by unipolar depression (Sanacora et al. 1999; Sanacora et al. 2004). GABAergic projections arising from the lateral preoptic area and the pontine ventral periaqueductal gray including the DRN itself strongly modulate the activity of the DRN serotonergic neurons during SWS and REM sleep, respectively (Gervasoni et al. 2000). Hence, it is possible, while speculative, that the alteration of the serotonergic function observed in CMS animals may be related to GABAergic dysfunction. Unfortunately, no direct measures of GABA have yet been reported in the CMS model.

A prolonged exposure to high level corticosterone (Mendelson and McEwen 1992) decreases the binding to 5-HT_{1A} receptors in the hippocampus. In addition,

there appears to be a direct relation between glucocorticoids and hippocampal 5-HT release (Linthorst et al. 2000, Farisse et al. 1999). Hippocampus is involved in the regulation of the HPA axis (De Kloet et al. 1998) being rich in corticosteroid and serotonin receptors (Uphouse 1997). We have not been able to detect any change in the level of corticosterone after CMS and this might parallel the findings of unchanged extracellular concentrations of 5-HT in hippocampus.

6.4 Individual variability

The exposure to CMS showed large response variability in the animals, indicating a need to consider individual differences in behavioral responses to CMS. Instead of considering CMS as a homogenous group, a correlation between different measures of depression-like symptoms were performed in Paper I, III and IV. Rats with lowest sucrose intake were those that spent more time in REM sleep, expressed highest number of REM sleep episodes, reduced their time in deep sleep, reduced their expression of p-CREB in dentate gyrus and showed highest level of 5-HT in SWS and REM sleep compared to waking. The analyses of these selective behavioral and molecular responses associated to stress-induced depression became more evident when this variability was specifically addressed. In our experience, correlation analysis is an important tool to identify CMS sensitive rats.

Inherited individual vulnerability to depression is a well known phenomenon (Kendler et al. 1994). Animals, humans included, differ in their capacity to cope with environmental challenges. Not all humans become depressed after exposure to stress, and not all cases of depression are a consequence of stressful experiences (Anisman and Zacharko 1992). An epidemiological study reports that life events and perceived strain are positively correlated to depressive symptomatology (Aneshensel and Stone 1982). The prevalence of depression has been reported to depend on the level of stress, ranging from 15 to 28% related to life events and 5 to 40% related to perceived strain (12 month and life-time prevalence, respectively). A predisposition for the stress-induced anhedonia has been indicated by submissive behavior in a prior resident-intruder test to CMS (Strekalova et al. 2004). In this view, high individual variability to CMS also reflects individual ability for coping with stress. We interpret

our results to reflect the individual response and the degree of coping with stressful situations.

7 CONCLUSIONS

1) The CMS model was successfully established in our laboratory. CMS rats consumed less sucrose (anhedonic response) compared to Controls. Saccharine intake did not change after CMS. Body weight was not affected by CMS.

2) CMS induced specific sleep changes e.g. increased REM sleep and sleep fragmentation. CMS increased activity in an open field test and decreased sexual activity (e.g. reduced capability to ejaculate).

3) CMS downregulated the expression of BDNF and p-CREB specifically in the dentate gyrus but not in the CA. No effect on total CREB level was found in the dentate gyrus or CA region.

4) CMS did not change the extracellular level of 5-HT in hippocampus.

5) Sucrose intake (reflecting anhedonia) correlated with REM sleep and SWS-2 alterations. Sucrose intake also correlated with p-CREB level in the dentate gyrus and hippocampal 5-HT level during in SWS and REM sleep. In other words, rats with lowest sucrose intake were those that spent more time in REM sleep, expressed highest number of REM sleep episodes, reduced their time in deep sleep, reduced their expression of p-CREB in dentate gyrus and showed highest level of 5-HT in SWS and REM sleep compared to waking.

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ERRATA

Paper IV, Discussion, page 16: “Serotonergic function is dependent on….. (Kandel, E.R., Schwartz, J.H., and Jessel, M.J., *Principles of Neural Science*. 4th ed, ed. J. Butler and Lebowitz, H. 2000: McGraw-Hill Companies, Inc).”, not “Serotonergic function is dependent on …. (REF).”