Towards a mouse model of depression

a psychoneuroendocrine approach

Sergiu Dalm

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“Men ought to know that from the human brain and from the human brain only arise our pleasures, joys, laughter, and jests as well as our sorrows, pains, grief and tears...It is the same thing which makes us mad or delirious, inspires us with dread and fear, whether by night or by day, brings us sleeplessness, inopportune mistakes, aimless anxiety, absent-mindedness and acts that are contrary to habit...”

Hippocrates

Voor Luka,
mijn zoon,
mijn waarheid

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List of Abbreviations

ACTH	adrenocorticotropic hormone
ADX	adrenalectomy
ANOVA	analysis of variance
AVP	arginine vasopressin
CA	cornu ammonis area of the hippocampus
CHB	circular hole board
CORT	corticosterone/cortisol
CRH	corticotrophin releasing hormone
DG	dentate gyrus area of the hippocampus
GLM	general linear model
GR	glucocorticoid receptor
HPA axis	hypothalamic-pituitary-adrenal axis
MIF	Mifepristone or RU38486, GR antagonist
Min	minutes
MR	mineralocorticoid receptor
OD	optical density
PCA	principal component analysis
PVN	hypothalamic paraventricular nucleus
SEM	standard of the mean
VEH	vehicle (=control)
RU	RU38486 (Mifepristone), GR antagonist
WM	water maze

Preface

Stress is an undeniable fact within modern societies. Our 24/7 economy challenge us with increasing social and professional pressures. Already in 2003, the World Health Organization declared “stress” as a major cause of health problems. Prolonged periods of stress that are out of control for the individual can lead to the development of mood disorders like depression. Patients are seriously hampered in day-to-day activities. As a consequence, this costs the society billions in terms of loss of productivity and health assurance costs. Discovery of new drug targets is a necessity.

A fundamental question for the neurobiology of mental health is how adaptation to both acute and chronic stress can become impaired and capable to precipitate emotional and cognitive disturbances characteristic for mood and anxiety disorders. The glucocorticoids, cortisol and corticosterone (CORT), are secreted from the adrenal glands by activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in response to stress, as well as in a circadian fashion. They are powerful hormonal neuroendocrine mediators of environmental influences on brain and body. Changes in the functionality of this glucocorticoid regulated stress system, supports the general believe that chronic stress leads to an altered hormonal secretion pattern, and is considered a central player in the development of mood disorders. Animal models for stress-related mood disorders are urgently needed for further development of new drugs.

In order to develop an animal model with altered functionality of the glucocorticoid regulated stress system we applied repeated exposure of mice to psychosocial stress, i.e., the presence of a rat. Studying mice in a familiar and novel environment(s) revealed a phenotype characteristic for chronic stress: emotional, cognitive processes were associated with dynamic changes in circadian patterns of neuroendocrine and behavioral activity during, and following the exposure to the chronic psychosocial stressor.

Our experimental designs allowed us to detect a differential contribution of brain systems to memory formation under stress. We found that a change in the sensitivity of the reward system contributes to cognitive impairments, which can be partially normalized by additional reward. Stress induced in mice by the chronic stress paradigm and stress in humans, induced a similar finding namely a shift to more rigid stimulus response learning. This indicates that our animal model can be used to study overlapping brain processes between the two species. The findings open a new perspective for the treatment of stress-related mood disorders like depression.

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Chapter 1

General Introduction

1. Introduction

Mood disorders such as major depressive- and bipolar disorder, share several characteristics (de Kloet et al. 2005): emotional changes related to approach/avoidance behavior, loss of interest or pleasure in daily activities (i.e. anhedonia), impairment of cognitive functions, reduced motor activity and alterations in the circadian pattern of physiological-, neuroendocrine- and behavioral responses (Endo and Shiraki 2000; Volkers et al. 2002; Keller et al. 2006). Chronic stress, specifically a dysregulation of the glucocorticoid system, is thought to be a precipitating factor in the etiology of depression.

The **main objective** of this thesis is to develop a mouse model that expresses signs and symptoms as seen with patients that suffer from depression, with special focus on the expression of anhedonia as the common denominator. Cognitive and emotional consequences of chronic psychosocial stress are studied with emphasis on changes in the responsiveness to positive stimuli. In addition, circadian patterns of neuroendocrine and behavioral activity are monitored in response to novelty, and within the familiar environment of the mouse's home cage.

The experiments are divided into three categories addressing:

1. Methodological optimization: since timing, context and duration of a stressor determine the experimental results, interference by unintentional stressors resulting from the experimental procedures has to be controlled and minimized. For example, separation of the stress effects induced by an injection from the action of the drug, and reduction in the adversity of the learning environment. We designed and optimized drug administration methods, and behavioral tests that minimized unwanted stress system activation (**Chapters 3, 4** and **5**).

2. Longitudinal studies in the home cages of mice, and in novel environments: these were conducted to measure circadian neuroendocrine activity, behavioral patterns, learning and memory, and emotional processes. Combined, the measurements will indicate whether anhedonia is expressed in our chronic stress model (**Chapters 2, 5, 6** and **7**).

3. Translational approach: chronically stressed mice, and chronically stressed healthy humans are subjected to comparable experimental designs that allow to test the use of distinct memory systems (**Chapter 8**).

Glucocorticoid functionality in mice was manipulated using two approaches:

1. Environmental challenges mimicking chronic psychological and psychosocial stress conditions in humans, namely: repeated, unpredictable and uncontrollable exposure of mice to rats (chronic 'rat stress').

2. Pharmacological intervention that compromises the functionality of the glucocorticoid receptor via repeated administration of the glucocorticoid receptor antagonist RU38486, also known as mifepristone (MIF).

The **goal** of the research described in this thesis is to characterize behavioral and neuroendocrine features in mice during, and in response to our chronic psychosocial stressor ('rat stress'), and during and after pharmacologically-induced dysfunction of the glucocorticoid receptor. We expect that the results will contribute to the understanding of the etiology of depression. Especially on the processes possibly underlying the expression of anhedonia, and may provide leads for alternative therapeutic approaches in humans.

2. Stress: Activity of the Hypothalamic-Pituitary-Adrenal axis

The original term *stress* was first used by Hans Selye for the biological phenomenon of a disrupted homeostasis (Selye 1937; Selye 1950). Since the 1950s, the definition of the term stress has evolved. **Box 1** describes the definition of stress against which the experimental designs in this thesis were created.

Box 1: Concept of stress

For operational use of the stress concept we favour the view of one of the pioneers in stress research, the late Seymour (Gig) Levine who defined 'stress' as a composite, multidimensional construct, in which three components interact: (i) *input*, when the stressor is perceived and appraised, (ii) *processing* of stressful information and (iii) *output* or stress response. The three components interact via complex self-regulating feedback loops with the goal to restore homeostasis through behavioral and physiological adaptations. These adaptations need to be coordinated in brain and body. The major communication systems, the autonomic nervous system and the HPA axis, are extremely important in this respect (Levine 2005).

Stressors that are of psychological nature occur due to uncertainty, lack of information and lack of control, and elicit the most profound neuroendocrine and behavioral responses. The ability to cope with such a psychological stressor is dependent on experience- and gene-related factors, and is affected by cognitive, non-cognitive and environmental inputs. Moreover, coping resources rely on the *context* in which the stressor is experienced. Powerful determinants of context are psychosocial factors such as social position, social support or attachment to a care giver. If any of these factors is disrupted - e.g., loss of control in a social environment, expulsion from social support, homelessness or deprivation of (maternal) care – an acute stressor may exceed the coping resources and produce strong emotional reactions, which ultimately may lead to a condition of chronic stress, exhaustion or burnout and enhanced vulnerability to mental diseases such as depression or anxiety disorders.

These modulations of the stress response have been defined by McEwen and Wingfield (McEwen and Wingfield 2003; McEwen and Wingfield 2010) as variations in an *allostatic state* that cumulatively strive towards homeostasis. *Allostasis* is defined as the process of achieving stability, or homeostasis, through physiological or behavioral change. In principle these changing allostatic states are adaptive, self-preservative and short-lasting. In terms of communication, successful *allostasis* (in establishing homeostasis) would mean that e.g., the HPA axis hormones involved are turned on rapidly when needed, and turned off efficiently when homeostasis has been achieved. The hormonal responses however may be inadequate, or excessive and prolonged and the cost to maintain homeostasis may become high. This leads to wear and tear, or *allostatic load*, ultimately enhancing the vulnerability to disease. Depression may be interpreted as a consequence of sustained hyperactivity of HPA axis activity resulting in excess circulating glucocorticoids.

The stress hormones cortisol and corticosterone (from here on abbreviated as 'CORT' respectively) follow a rhythmic secretion pattern. They are secreted in hourly pulses, exhibit a circadian pattern and can be induced by stressors superimposed on the rhythmic secretion (De Kloet et al. 1998; Windle et al. 1998b). These modes of CORT secretion are regulated by inputs from the suprachiasmatic nucleus, paraventricular nucleus of the hypothalamus, prefrontal cortex, amygdala, and hippocampus, among others. Both stress-induced changes in CORT levels and circadian patterns of CORT will be addressed in this thesis. Following, the concentration of CORT will be our marker indicating stress system activation as a result of our experimental procedures, which is controlled by the Hypothalamic-Pituitary-Adrenal (HPA) axis (see Figure 1 in section 2.1).

2.1. Stress system activation

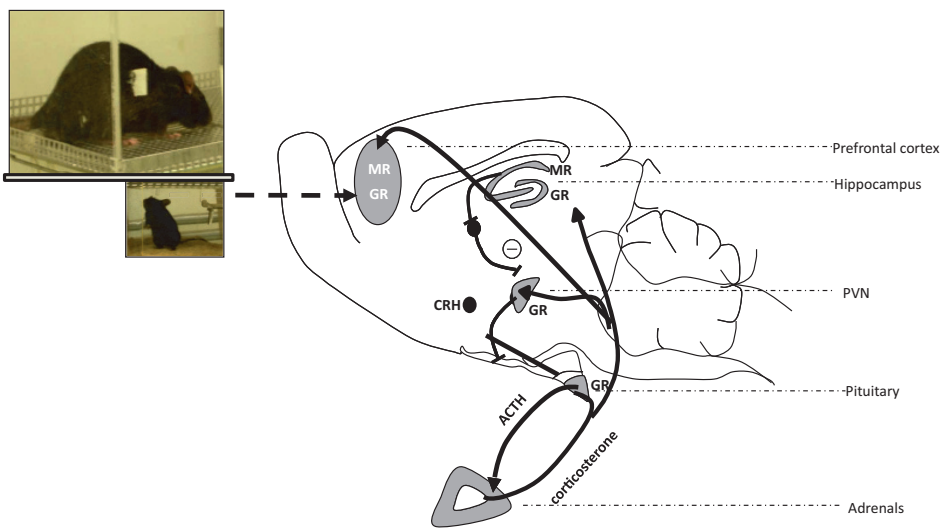
To efficiently cope with threatening situations, the organism requires a set of emotional, behavioral and neuroendocrine responses, summarized as the *stress response*. The stress response is an essential component of the natural defense/response mechanism, providing energy resources in order to react in the most efficient way for the organism. Ultimately, the stress response allows the organism to integrate new with previously learned response strategies, often leading to a new set point of psychological and biological reactivity. However, stress, especially chronic stress, is predominantly

associated with a negative emotional state. As will be described in the following sections, a period of stress may become deleterious when it remains uncontrollable.

The origin of stressors can be *systemic,* directly disturbing physiological integrity (e.g., infections, temperature or blood volume changes) and *psychological* or *psychosocial* (e.g., social conflict, traumatic life event); both able to disturb mental integrity. Exposure to a demanding, threatening event either real or imagined will result in a freeze, fight or flight stress response. This response is governed by two main systems that process the perceived information into a reaction. First, the rapid activation of the sympathetic nervous system increases the release of catecholamines: adrenaline and noradrenaline. These catecholamines stimulate the peripheral organs and increase the blood flow to the central nervous system and muscles within seconds. This allows the organism to promptly respond to the stressor with heightened arousal and attention. The second, slower regulatory response is activation of the HPA axis, characterized by secretion of the glucocorticoid hormones (mainly cortisol in humans, corticosterone in rats and mice (De Kloet et al. 1998; de Kloet et al. 2005).

Activation of the HPA axis by a stressor (see Figure 1) rapidly induces the parvocellular neurons of the paraventricular nucleus of the hypothalamus (PVN), to secrete corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) in the portal vessel system; the portal system being the vascular link between the

Figure 1 **Hypothalamic-Pituitary-Adrenal axis**



hypothalamus and the anterior pituitary. Within the anterior pituitary, CRH stimulates cells to synthesize adrenocorticotrophin hormone (ACTH) from its precursor pro-opiomelanocortin (POMC). AVP potentiates the effect of CRH, leading to more release of ACTH. Subsequent increases in circulating ACTH then drive synthesis in and secretion of CORT from the adrenal cortex into the blood.

CORT serves a wide variety of functions in the body. They enhance catabolism, mobilizing lipid and glucose reserves, suppress the immune system and increase the cardiovascular tone (Munck and Naray-Fejes-Toth 1994; De Kloet et al. 1998). In addition, CORT regulates their own secretion by facilitating recovery and inhibiting HPA axis activity. This negative feedback is exerted at several levels of the HPA axis that are activated by the given stressor, thereby normalizing the activity of the stress system and preventing it from overshooting. HPA axis activation enables the organism to respond with the required energy resources to meet the demands of the event.

Prominent in the brain's stress circuitry are the amygdala nuclei for regulation of emotional responses (McGaugh 2004; Phelps and LeDoux 2005), the hippocampus (which defines context in terms of time and place) for learning and memory processes (Sanders et al. 2003) and prefrontal cortex regions for planning and control of actions. Depending on the magnitude of CORT signaling i.e., non-stressed, acute- or chronic stress, the functionality of these brain systems will be affected and thereby alter neuroendocrine-, as well as emotional and cognitive processes (Quirk and Beer 2006; Oitzl et al. 2010), while also changing the circadian pattern of HPA axis activity. The latter will be addressed in the next section.

2.2. Circadian pattern of HPA axis activity

The daily pulses in glucocorticoid concentration follow a circadian rhythm in blood plasma. This rhythm is characterized by peak concentrations of CORT and ACTH at the start of the active period, which is early in the morning for diurnal animals like humans (Krieger et al. 1971; Steiger 2003), and at the onset of darkness for nocturnal animals like rats and mice (Windle et al. 1998a; Windle et al. 1998b; Barriga et al. 2001); lower concentrations occur during the course of the day/night.

Depending on when a stressor is applied during the phase of the rhythm, the amplitude and duration of the stress response differs (Young et al. 2004). Underlying the circadian rhythm, CORT secretion exhibits an ultradian rhythm which is characterized by approximately hourly bursts (de Kloet and Sarabdjitsingh 2008; de Kloet 2009). These circadian and ultradian rhythms of CORT are also expressed in the brain (Droste et al. 2009), and aimed to prepare the organism for environmental changes ahead, i.e., light-

dark cycle and foraging activity. Daily variations in CORT secretion are thought to be fundamental for the maintenance of physiology and well being. Disturbances in the normal secretion pattern, for instance due to chronic stress, are considered to enhance vulnerability to stress-related disorders (Young et al. 2004; de Kloet et al. 2005).

Dramatic changes in circadian patterns of glucocorticoids hormones have been described in aging and psychiatric disorders like depression and Alzheimer's disease (Hatfield et al. 2004; Peeters et al. 2004). The excessive activity of the HPA axis is generally associated with impaired mental and physical health (Sapolsky 1999; Lupien and Wan 2004) and characterized by increased basal and/or stress-induced levels of glucocorticoids and ACTH (Van Eekelen et al. 1995; Herman et al. 2001).

Although mouse models for a wide range of human stress-related disorders have been developed, surprisingly little is known about the impact of age, chronic stress, and repeated blockade of glucocorticoid receptors on basal regulation subserving circadian activity of the HPA axis in mice. All this factors are of importance to further understand how chronic stress can precipitate the development of stress-related disorders, like depression. In this thesis, we will focus on the circadian patterns of neuroendocrine and behavioral activity during and after chronic stress, in mice. In the following sections the role of the glucocorticoid receptors in stress system regulation is introduced.

2.3. Mineralo- and Glucocorticoid receptors

The actions exerted by CORT depend on the functionality of two brain nuclear receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The pharmacological properties, distribution pattern, and function are distinct for MR and GR (De Kloet et al. 1991; Veldhuis et al. 1992; McEwen 1996; De Kloet et al. 1998; Oitzl et al. 2010).

MR has a 10-fold higher affinity for the naturally occurring CORT than GR (Kd = 0.5 and 5.0 nM, respectively (De Kloet and Reul 1987). Consequently, MR is almost fully saturated at low circulating levels of CORT, whereas GR becomes occupied at increasing levels of CORT as seen during stress and the circadian peak. MR expression in the brain is more restricted to certain areas, with the highest density in hippocampus, and to a lesser extent in the amygdala, septum, PVN and brain stem. GR is expressed throughout the brain (De Kloet et al. 1998), with high expression in the hippocampus, septum and parvocellular part of the PVN of the hypothalamus, brain stem; moderate levels are reported in the central amygdala.

Upon binding of CORT to MR and GR a complex is formed. The corticosteroid-receptor-complex dissociates from a large protein-complex and translocates from

the cytosol to the nucleus as homodimers (MR/MR) or heterodimers (MR/GR). In the nucleus, the dimers bind to glucocorticoid response elements (GREs) in the promotor areas of genes, where they recruit components of transcriptional machinery and activate transcription (McEwen et al. 1986; De Kloet et al. 1991; Morsink et al. 2007). MR and GR can enhance (transactivation) or repress (transrepression) gene expression (Truss and Beato 1993; Beato et al. 1996), and thus influence target genes that are involved in the emotional, behavioral and neuroendocrine response.

MR and GR mediate different aspects of CORT signaling. Studies have emphasized the critical functionality of MR in the sensitivity and feedback of neuroendocrine responses at all stages: (i) primarily the binding of CORT to MR controls the release of ACTH during both the circadian trough and peak (Dallman et al. 1989; Ratka et al. 1989; Bradbury et al. 1994); (ii) blockade of MR by a specific antagonist increases the level of circulating CORT under basal, resting conditions and in response to novelty stress (Ratka et al. 1989). It was concluded that one of the MR-mediated effects of CORT is the initial constraint of HPA axis activity (Oitzl et al. 1995). After acute stress, MR mRNA is quickly upregulated via CRH which is associated with increased inhibition of HPA axis activity, leading to normalization of the disturbance (Hugin-Flores et al. 2003). In hippocampus, MR activation maintains excitability, while GR occupancy suppresses excitability, which is transiently raised by excitatory stimuli. MR and GR distinctively mediate the actions of CORT secretion and its effects throughout the day. Thus, MR activation by CORT maintains basal activity of the HPA axis and controls the sensitivity or threshold of the system's stress response, known as the "proactive" mode. MR promotes coordination of circadian events (e.g., sleep/wake cycle, food intake) and is involved in processes underlying selective attention, integration of sensory information and response selection (Oitzl and de Kloet 1992; Oitzl et al. 1995).

In the second "reactive" mode, when CORT concentrations increase as a result of circadian rhythm and stress, GR becomes activated. GR activation will terminate HPA axis activation via negative feedback leading to reduction in CORT concentration. GR feedback takes place in different brain sites including the pituitary and PVN (Dallman et al. 1987; Levin et al. 1988). GR activation enables an organism to incorporate the neuroendocrine and behavioral responses deployed by facilitating learning and memory processes (De Kloet et al. 1998).

As described, MR and GR are expressed in brain regions involved in emotional, cognitive and neuroendocrine regulation. The receptors mediate rapid mono-genomic CORT actions within seconds to minutes, until the slow and long lasting genomic actions start after an hour lasting hours to days. In this thesis, we set out to alter HPA axis activity

by activation or blockade of the corticosteroid receptors, using either an environmental stressor and/or pharmacological manipulation with a GR blocker. Subsequently, we expect to find alterations in emotional, cognitive and neuroendocrine regulation as indicators of symptoms as seen with depression. Next, the role of MR and GR in the domain of emotional and cognitive processes is discussed.

2.4. Hypotheses of glucocorticoid action and cognition

Aberrant CORT concentrations as seen during periods of chronic stress are causally related with hippocampal, prefrontal cortex and amygdala dysfunction. However, the underlying mechanism is still unclear. Next, we will describe two hypotheses that provide clues to the underlying mechanisms.

The glucocorticoid cascade hypothesis (Sapolsky 1992; Sapolsky 1999): The elevated CORT is believed to arise from a GR dysfunction. When GR function is normal, the rise in CORT concentrations is terminated following GR activation. However, in patients that suffer from a mood disorder like psychotic major depression where CORT levels remain elevated, reduced GR expression in brain (Webster et al. 2002) and in peripheral tissue (Gormley et al. 1985; Pariante 2006) is found. The GR reduction weakens the negative feedback action and induces CORT resistance (De Kloet et al. 1997; Pariante et al. 2004; Ridder et al. 2005). As a result of decreased GR expression or function, circulating levels of CORT are elevated as a compensatory reaction to overcome the CORT resistance at the GR (Sapolsky et al. 1986; Pariante 2003). Prolonged hypersecretion of CORT damages brain structures essential for HPA axis functioning e.g., hippocampus, prefrontal cortex and amygdala. Following, the reduced functioning of brain structures leads to a feed-forward circuit in which ongoing stressors drive overproduction of CORT.

An important role for GR in control of aberrant CORT concentrations is apparent. However, next to GR the existence of another corticosteroid receptor was proven: the MR (Reul and de Kloet 1985).

The MR-GR balance hypothesis is based on (dys)functioning of either one or both receptors, creating an imbalance in MR-GR activation in context with the event. Whereas MR operates in pro-active mode to prevent homeostatic disturbance, additional GR activation promotes the reactive recovery after stress and following circadian peaks (Oitzl and de Kloet 1992; de Kloet et al. 1993a). MR and GR activation in the context of an event facilitate learning and memory, whilst MR and GR activation out of context impair memory (Joels et al. 2006). Studies with transgenic MR and GR mouse models show that

overexpression or inactivation of either two receptors seriously impair physiological and psychological responses to stress (Gass et al. 2001).

MR and GR are co-expressed in most cells of the hippocampus (van Steensel et al. 1996). The hippocampus is a key structure for learning and memory processes, and stress response regulation in general. Processing of spatial information can be specifically modified depending on activation of MR and GR. GR facilitates consolidation of the employed behavioral response. It is evident that dysfunction of MR and GR signaling may have profound effects on emotional, behavioral and neuroendocrine responses.

Blockade of MR activation with antagonist or genetic deletion of forebrain MR, interfered with memory formation (Zhou et al. 2010), and also chronic MR activation impairs spatial memory (Douma et al. 1998; Yau et al. 1999). Furthermore, MR affects emotional behavior. Predominant MR activation alters the behavioral response in novel situations and subsequent explorative search patterns, influencing what is learned and memorized (Oitzl and de Kloet 1992; Zhou et al. 2010). Blockade of MR results in an increased exploration on the elevated plus-maze (time spent in open arms indicates that animal is less anxious), which can be interpreted as an anxiolytic effect (Korte et al. 1996; Smythe et al. 1997; Bitran et al. 1998).

GR promotes memory processes and facilitates consolidation of a learned behavioral response. Mice with alterations in GR functionality, either by a mutation of the GR (e.g., GR-knockout, GR $^{dim/dim}$ mice) or by pharmacological intervention (treated with a GR antagonist intracerobroventricularly), showed impaired spatial memory. In addition, GR activation affects anxiety related behavior, with reduced anxiety in conditions of decreased GR functionality (Tronche et al. 1999; Jakovcevski et al. 2008). Since GR blockade interferes with anxiety motivated behavior, this can be considered an anxiolytic effect, as demonstrated by Korte and colleagues (Korte et al. 1996).

An imbalance in MR or GR activation, due to genetic, environmental, and/or pharmacological intervention is thus suggested to underlie the emotional, behavioral and neuroendocrine disturbances that make the organism more vulnerable for stress related mood disorders like depression (De Kloet et al. 1998; Brinks et al. 2007c; Oitzl et al. 2010).

The experiments described in this thesis aimed to modulate the activity of the glucocorticoid stress system, thereby changing the pattern of MR and GR activation. The psychoneuroendocrine effects were assessed before, during and after onset of chronic stress, and following GR antagonist administration.

2.5. Stress, learning and memory

Memory formation is modulated by task-inherent appetitive and aversive characteristics. Other stimuli occurring in close context with the task can impair or enhance memory (Dawson and McGaugh 1971; McGaugh et al. 1972). These stimuli can be either negative stressors or positively rewarding. The learning and memory process can be described as follows. When a situation is encountered, gain of information (acquisition) about the event takes place. During and directly after the event (Joels et al. 2006), a memory trace of the gathered information and response selection is created and stabilized (consolidation). Upon return to a similar situation the previously acquired response selection is retrieved and either used to deal with the situation at hand, or the response is modified as a result of environmental and cognitive stimuli (retrieval).

The impact of stress on learning and memory processes is described as being impairing, improving or even apparently ineffective (see for extensive review: (Joels et al. 2006; de Quervain et al. 2009; Conrad 2010). Several parameters are important to notice: (i) context – close association between the stress and the learning task facilitates performance. Extremely low or high CORT concentrations in close-context impair performance, demonstrating the inverted U-shaped dose-effect curve of CORT; (ii) convergence in time - stress hormones present around the time of learning and retrieval, i.e., during the actual performance of the behavioral task, can facilitate learning. However, high concentrations of stress hormones before or after learning impair performance; (iii) stressor specificity – different stressors activate different and overlapping brain regions. Whereas physical stressors activate lower brain regions and ascending pathways into the forebrain (e.g., regions involved in pain responses), psychological stressors activate the higher brain regions (hippocampus, prefrontal cortex, amygdala); (iv) frequency of stressor occurrence – single or repeated exposure to a stressor. Characteristics of the stressor, context, timing, memory phases (acquisition, consolidation, retention) during which stress is experienced are important variables contributing to the effect of stress on cognition. In addition, age and gender effects are known. Moreover, there is considerable individual variation in the effects of stress due to genetic background and life history.

Stress can shape the memory trace and subsequent response during future encounters by modifying learning and memory processes that occur before, during and after an initial event. These effects exerted by stress operate in brain circuits that primarily were pronounced by genetic and experience-related factors in preparation of upcoming events.

In order to study the full range of stress effects on learning and memory processes, we designed and optimized learning and memory tasks (**Chapter 5**). In

addition, we were able to perform a unique translational study from mouse to human (**Chapter 8**), where we tested the impact of chronic stress on the use of different memory systems. The following section will give a short impression on different memory systems.

2.5.1. Memory systems

Memory systems differ regarding the kind of information they process, the performed operations and the underlying neural structures (Gabrieli 1998; Squire 2004b). Researchers predominantly focused on the stressor and its impact on memory, but have rather neglected that memory consists of multiple systems processing information in parallel (Squire 2004a; Squire et al. 2004b). For example, changing catecholaminergic activity in the amygdala, a brain structure involved in emotional memory, (Cahill et al. 1995) can modify hippocampal spatial memory (de Quervain et al. 2009; Roozendaal et al. 2009).

Interactions between memory systems are most evident in situations in which multiple memory systems can support behavioral performance. The well-known water maze (Morris 1984) task where rodents navigate to find a platform provides an example. When the platform is visible and in a fixed location, the performance can rely on both hippocampus-dependent spatial (''cognitive'') and neostriatum-dependent stimulus-response (stimulus-response: S-R; ''habit'') memory. In an elegant study, Kim et al (Kim et al. 2001) demonstrated in rats that stress prior to training facilitated the use of an S-R strategy and reduced the use of a spatial strategy to find the platform. Spatial memory, which is considered to rely on more complex processes than S-R memory, supports the acquisition of flexible, consciously accessible knowledge (such as the event of your birthday) that is particularly ascribed to the hippocampus (Scoville and Milner 1957; Eichenbaum 2004). Non-spatial S-R learning process associations, such as ''stop your car when the traffic lights are red", are not necessarily accessible to consciousness and relies on the caudate nucleus (Knowlton et al. 1996; Jog et al. 1999). The two memory systems can work in parallel and process information simultaneously. Cognitive tasks can be designed that allow a differential use of spatial and non-spatial memory systems.

Acute stress prior to training in a task that could be acquired by a hippocampus-based spatial, and a caudate-based non-spatial S-R strategy resulted in predominantly caudate-based learning both in rodents and humans (Kim et al. 2001; Packard and Wingard 2004; Schwabe et al. 2007). This stress-induced modulation of hippocampus-based and caudate-based learning and memory systems is assumed to be influenced by the amygdala as well (Packard and Wingard 2004). Emotional components (including anxiety, punishment, reward) are included in the majority of behavioral tasks for rodents.

Stress before the learning task affects the performance, which might be due to the differential use of memory systems. Psychosocial stress before training in an instrumental task rendered the participants' behavior insensitive to the change in the value of a reward: i.e., stress led to habit non-spatial performance at the expense of goal-directed spatial performance in humans (Schwabe and Wolf 2009). This study proves that the recognition of change in the rewarding values of stimuli is differentially perceived under stress.

The modulation of non-spatial habit, and spatial cognitive memory systems by stress has attracted a lot of scientific attention the past decade (Kim et al. 2001; Packard and Wingard 2004; Schwabe et al. 2007; Dias-Ferreira et al. 2009). However, the effects of prolonged or repeated periods of stress on the modulation of these two memory systems have not been described. In this thesis we determined the impact of chronic stress in both mice and humans. The results could indicate the underlying processes that drive behavioral alterations as seen in patients that suffer from depression.

2.6. Depression: emotional and cognitive disturbances

Depression is characterized by several symptoms (see **Box 2**).

Box 2: Symptoms of depression

The Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV-TR), describes that at least five of the following signs and symptoms must be present for at least 2 weeks as to be characterized as a depressive disorder: (1) anhedonia: loss of interest for or inability to experience pleasurable emotions from normally pleasurable life events, (2) appetite/weight disturbances, (3) sleep disturbance / circadian activity pattern, (4) psychomotor retardation, (5) loss of energy, (6) feelings of depressed mood, (7) worthlessness/guilt, (8) concentration difficulties/ indecisiveness and (9) thoughts of death/suicide. It is recognized that the latter five symptoms are typical human characteristics and cannot be modeled in mice. Our experimental setup described in this thesis aimed to induce symptoms (1), (2), (3), (4) and (8) using our 'rat stress' paradigm in mice.

The core symptom of depression is anhedonia, which is defined as the inability to experience pleasure (Ribot 1897). Anhedonia is indicative for alterations in the perception of emotional and other environmental stimuli, which in turn affects cognitive processing, and vice versa. In psychopathology disturbances in the detection, response to, and interpretation of emotions are common, and can produce altered emotional responses.

Patients suffering from psychotic major depression show reduced emotional reactivity. More specifically, a deficit in processing of positive stimuli is evident, while a bias towards the perception of negative stimuli exists. This imbalance in emotional processing results in depressed mood and anhedonia (Phillips et al. 2003; Leppanen 2006; Bermpohl et al. 2009). However, the neural substrates for mood disorders are poorly understood. Next to disturbances in limbic and prefrontal brain regions, alterations in the brain reward mechanism (the mesolimbic dopamine system) are likely. Neuroimaging studies show reduction in hippocampal volume, and alterations in prefrontal cortex, amygdala and brain regions associated with the mesolimbic dopamine system (i.e., nucleus accumbens and the ventral tegmental area; (Nestler and Carlezon 2006; Martin-Soelch 2009). Studies in depressed patients revealed a decrease in reward sensitivity toward positive stimuli (Shankman et al. 2007) and altered reward-related decision making (Forbes et al. 2007) functions are restored following classic antidepressant treatment in a subset of patients (Drevets 2000). Disturbances in emotional processing affect cognitive processing, like memory formation (Stiedl et al. 2000) which in turn affects the emotional response to stimuli (Blair et al. 2007). Depending on the CORT concentration, and subsequent binding to MR and GR, emotional and cognitive processes can be modulated (Brinks et al. 2007b).

Patients suffering from depression exhibit hyperactivity of the HPA axis even before the onset of clinical symptoms. The CORT concentrations are elevated during the circadian cycle (Keck and Holsboer 2001). Remarkably, patients with a severe form of depression (psychotic major depression) appear to be relieved of symptoms following treatment with the GR antagonist MIF (Belanoff et al. 2001a; DeBattista and Belanoff 2006). Diagnostic and Statistical Manual of Mental Disorders 4th edition (DSM-IV-TR), describes that at least five of the signs and symptoms referred to in **Box 2** must be present for at least 2 weeks as to be characterized as a depressive disorder. It is recognized that symptoms (5), (6), (7) and (9) are typical human characteristics and cannot be modeled in mice. Our experimental setup described in this thesis aimed to induce signs and symptoms (1), (2), (3), (4) and (8), using our 'rat stress' paradigm in mice.

2.6.1. Treatment of depression

The classic antidepressants (e.g., tricyclic antidepressants; Serotonin and Noradrenaline Reuptake Inhibitors: SSRI, SNRI) have shown therapeutic effect. However, the therapeutic effect may take weeks before being expressed, with increased risk for the patient to experience unwanted side effects, and the increased likelihood that the risk on suicide remains high during the first weeks of treatment (Schatzberg 2002). Hence, new antidepressants are warranted. We know that the classic antidepressants affect MR and GR expression in brain, and normalize CORT secretion patterns (Reul et al. 1993). The normalization was thought to be partly due to restoring the negative feedback mechanism at the level of GR (Ribeiro et al. 1993; Heuser et al. 1996; Pariante and Miller 2001). Thus, targeting the receptors that mediate CORT secretion, MR and GR, might open up potential new drug treatment for patients that suffer from depression.

Indeed, clinical trials revealed that high doses (600 - 1200mg/day) of the GR antagonist mifepristone (i.e., RU38486) show therapeutic efficacy for the most severe form of depression, psychotic major depression (Murphy et al. 1993; Belanoff et al. 2001a; DeBattista et al. 2006). Treatment for several days only, already improved emotional and cognitive processes, together with restoration of aberrant levels of CORT. The 'antidepressant' effect is thought to arise via the following pathway: GR antagonism leads to increased amplitude in pulsatile and circadian CORT levels, which induce a resetting of the HPA axis activity, with a subsequent change in GR sensitivity, and a distinct action via MR by CORT (Sartor and Cutler 1996; De Kloet et al. 1998). The rhythmicity of the circadian activity is enhanced (van Haarst et al. 1996). In addition, GR resistance could be compensated via increased MR expression (Wodarz et al. 1992; Calfa et al. 2003).

Additional evidence for GR dysfunction comes from depressed patients that received the GR agonist dexamethasone. These patients showed non-suppression of ACTH and CORT (Nemeroff 1996; DeBattista et al. 2006), suggestive for an impaired negative feedback at the level of GR. It has become clear that GR dysfunction is associated with stress-related psychiatric disorders. This shift in the balance of MR and GR activation renders the organism more vulnerable to diseases (Holsboer 2000; de Kloet et al. 2005).

2.6.2. Effects of GR antagonism

The role of GR has been studied specifically by pharmacological modulation, using the GR antagonist RU38486 (Roussel-Uclaf 38486; first synthesized in 1981) also known as mifepristone or in short RU486. It has both antiglucocorticoid and antiprogesterone

activity. The latter is utilized in early termination of pregnancy. RU38486 is readily absorbed via the oral route in humans and rodents. The α1-acid glycoprotein binds RU38486 in humans, increasing its bioavailability (Agarwal 1996). However, the bioavailability of RU38486 in rodent is 40% partly because the rodent's α1-acid glycoprotein does not bind RU38486 explaining the low levels in plasma and fast plasma clearance (Philibert and Teutsch 1990). RU38486 is distributed to all tissues, thereby exerting a generalized antiglucocorticoid activity (Heikinheimo and Kekkonen 1993). Intracerebroventricular (ICV) administration of RU38486 was performed. GR antagonism does not interfere with basal resting activity of the HPA axis at the trough of circadian activity. However, RU38486 increases the circadian peak secretion of CORT and prolongs stress induced activity (Gaillard et al. 1984; Ratka et al. 1989; van Haarst et al. 1996).

GR antagonism has been shown to protect mice and rats against the negative impact of high CORT and chronic stress on hippocampal functioning. Mice with streptozotocin-induced type I diabetes and high CORT for eleven days showed hippocampal alterations; treatment with mifepristone for 4 days during the early phase of diabetes attenuated the morphological signs and protected the mice from cognitive deficits (Revsin et al. 2009). Neurogenesis was normalized in rats that underwent a chronic stress paradigm for 21 days, and were treated with mifepristone during the last 4 days (Mayer et al. 2006; Oomen et al. 2007). The protecting, and therapeutic efficacy of GR antagonism is most pronounced in conditions of high CORT levels. Although CORT concentration increases due to GR antagonism, there is no receptor to act on. It thus appears that the resulting shift in MR-GR activation is responsible for the positive effects of GR antagonism.

Taken together, the GR antagonist mifepristone (MIF) increases HPA axis responsiveness and resilience in humans (Lamberts et al. 1991). Similar effects are found in rats (van Haarst et al. 1996). Whereas much is already known on GR functioning, the mechanism underlying the apparent therapeutic efficacy of GR antagonism is unclear. Before we would study the impact of GR antagonism in our chronic stress model we will determine the effects of GR antagonism in naive mice. We will collect data related to circadian HPA axis activity, emotional, behavioral and neuroendocrine responses (**Chapter 4**). This data will provide parameters that can show whether in stressed mice similar processes are affected and the possible protecting/normalizing effects of MIF on those processes.

3. Rodent models of depression

Animal models of depression can be of genetic origin, induced by (social-) environmental challenges (usually exposure of rodents to various types of stressors) or via pharmacological modulation. The resulting neuroendocrine and behavioral changes are indicative for certain signs and symptoms (Willner 1990; Willner et al. 1992; Willner 1995). Chronic stress is believed to render the organism more vulnerable to the development of stress related psychiatric disorders. Therefore, most animal models use long-term manipulations of the stress system to model the predisposition to depression (Willner and Mitchell 2002). Although the subtypes of depression are typical human disorders, a subset of human characteristics can be assessed in animal models; see Table 1.

Table 1: Symptoms associated with depression in humans and reference to Chapters in the thesis that determined the expression of the human-like symptoms in our mouse model for depression.

Symptoms of depression*	Measurable in animal models?	Determined in Chapter
Anhedonia	Yes	6 and 7
Weight changes	Yes	6
Sleep disturbances/circadian activity pattern	Yes	6 and 7
Psychomotor retardation	Yes	6 and 7
Fatigue/loss of energy	Yes	not determined
Depressed mood	No	n.a.
Feelings of worthlessness/guilt	No	n.a.
Diminished ability to think/make decisions	Yes	6, 7 and 8
Thoughts of death/suicide	No	n.a.

**Source: Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV, American Psychiatric Association 1994.*

Table 1 indicates which human characteristics were determined in our chronic stress model. A valid model of depression would ask for multiple symptoms to be induced and measured (see also **Box 3**). Preferably, multiple behavioral tests need to be performed to approximate the characteristic mood symptoms (Anisman and Matheson 2005).

First, a brief introduction to rodent models for human stress, primarily targeting GR and MR is provided, followed by a description of our chronic 'rat stress' paradigm in more detail.

The pivotal role of GR for survival has been shown in mice with a total deletion of GR ($GR^{null/null}$). Ninety-five percent of these mice died shortly after birth because of impaired lung development (Cole et al. 1995; Reichardt and Schutz 1996). The remaining 5% survived because of an incomplete knockout of the GR. However, partial inactivation of the GR produced depression-like changes in behavior and a mild HPA axis dysregulation. Anxiety-associated locomotor activity was increased and adrenal responsiveness was augmented. This occurred in GR heterozygous mice (GR+/- with a 50% reduction of GR; (Ridder et al. 2005; Chourbaji and Gass 2008) and in mice with postnatally induced deficiency of GR in the forebrain (Boyle et al. 2005; Boyle et al. 2006). A reference to the variety of genetically modified GR mouse models can be found in (Muller et al. 2002; Urani and Gass 2003; Kolber et al. 2008).

Box 3: A model is defined as any experimental preparation serving the purpose of studying a condition in the same or different species. In developing and assessing an animal model, it is critical to consider the explicit purpose intended for the model, to determine the criteria required to establish its validity. Validation of models for psychiatric disorders include consideration of the following: *construct validity* (theoretical rationale for designing the model based on clinical expression of the disorder); *face validity* (phenomological similarity between the model and the disorder); *predictive validity* (the correspondence between drug actions in the model and the clinical setting; (Willner 1997; Bloom and Kupfer 2001). It depends on the scientific question addressed which animal model is to be used (e.g., social stress paradigms with or without physical contact).

Numerous animal models of 'depression' are available that predominantly focus on the expression of negative emotions. We set out to develop a chronic stress paradigm that would allow investigation of immediate and long-term consequences for emotional and cognitive responses, in relation to positive rewarding stimuli and stress system activity patterns. In addition, behavioral measurements were designed to cover a wide range of effects.

We aimed to disturb the MR-GR balance in two ways, using (1) an environmental challenge –chronic 'rat stress' - to modulate the activity of the stress system over a longer

period, and (2) pharmacological modulation of GR activity using the GR antagonist mifepristone. Neuroendocrine, emotional, cognitive and behavioral patterns were assessed. The next section provides more detail on the features of our chronic stress model.

3.1. Environmental stress paradigms

One of the precipitating factors in the development of depression is a disturbed reactivity to novel situations. This reactivity is a combination between genetic predisposition and past learning experiences. In humans, chronic psychological stress during adulthood can precipitate psychiatric disorders (Corcoran et al. 2003). Central features of chronic psychological and psychosocial stressors in humans are repeated, unpredictable and uncontrollable exposure to (or imagination of) threatening situations. To mimic these central features, animal models are based on social confrontations with or without physical contact (Apfelbach et al. 2005). To clarify the difference between a stressor with- and without the ability of physical contact, an example for each is described below.

3.1.1. Social stress paradigm with physical contact

Chronic stress in mice can be induced by social defeat. A mouse is rendered subordinate by repeated exposures and defeat by a dominant mouse during several weeks. Consequences are: decreased locomotor and exploratory activity, increased anxiety. HPA axis activity is affected as indicated by low body weight, elevated CORT and ACTH concentrations, and low hippocampal MR mRNA expression (Koolhaas et al. 1997) (Koolhaas et al. 1997; Veenema et al. 2003). Schmidt et al., developed a chronic social stress paradigm where mice are exposed to different cage members every 3 - 4 days, which creates an unstable social hierarchy; an unavoidable stressor. The consequences are expressed by increased adrenal and reduced thymus weight, flattened circulating circadian CORT concentrations patterns, reduced mRNA expression of hippocampal MR and GR, increased expression of AVP in the PVN, increased anxiety and lower responsivity to a sucrose solution (Schmidt et al. 2007; Schmidt et al. 2008; Sterlemann et al. 2008). However, these stress paradigms are a mix of physical and psychological stressors. Whereas physical stressors affect predominantly lower brain areas (e.g., brain stem) that subsequently affect the forebrain, psychological stressors are processed in higher brain areas (e.g., prefrontal cortex, amygdala, and hippocampus).

Psychological and psychosocial stressors are ethologically more relevant compared to physical stressors, and resemble the kind of stress that is related to

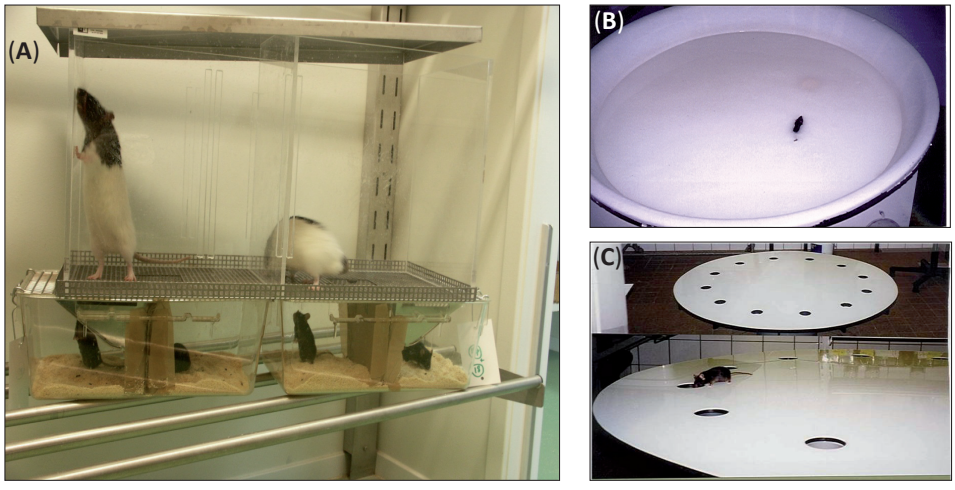
depression in humans (Calvo-Torrent et al. 1999; Apfelbach et al. 2005; Beekman et al. 2005). In rodents, the behavioral effect of predator exposure becomes manifested in the defeated subject as increased anxiety-like behavior, risk-assessment in novel environments and learning and memory impairments (Calvo-Torrent et al. 1999; Grootendorst et al. 2001a; Grootendorst et al. 2001b; Adamec et al. 2004; Diamond et al. 2006). For our stress model we induce chronic psychosocial stress by means of exposing mice to the presence of a rat, without physical contact.

3.1.2. Social stress paradigm without physical contact

Already sensory stimuli (visual, auditory and olfactory) are sufficient to activate the stress system associated with the release of CORT (Blanchard et al. 1998; Diamond et al. 1999; Linthorst et al. 2000; Beekman et al. 2005). In nature, mice and rats avoid each other and it was shown that exposure of mice to rats in a laboratory setting increased CORT concentrations in blood plasma and in the extracellular fluid of the mouse brain, as measured using microdialysis (Linthorst et al. 2000). Previously, our group created a chronic stress model for mice by exposing mice repeatedly to the presence of a rat, a procedure referred to as chronic 'rat stress' (Figure 2A).

Mice and rats could hear, see and smell each other, without physical contact (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Acute and some long-term effects on neuroendocrine and behavioral responses are evident in the mice during chronic 'rat stress'. Using two distinct spatial learning tasks, we showed that chronic 'rat stress' impaired learning and memory performance in C57BL/6J mice. More specifically,

Figure 2



stressed mice used a different strategy to locate the escape platform (water maze; Figure 2B) or exit hole (circular hole board; Figure 2C) in either two tasks. In addition, immediately after the 9th rat exposure and one week after the last rat exposure, the plasma CORT concentration was increased. Interestingly, three months after cessation of the stressor, stressed mice displayed a different behavioral response after being placed in the dark compartment of the light-dark box. Stressed mice were more active in the light compartment. This is opposite to their natural preference namely, seeking shelter in the dark area of the environment (Grootendorst et al. 2001a; Grootendorst et al. 2001b).

The effects of chronic stress in animal models are mainly assessed in short-lasting test-situations involving additional novelty stress. Less is known about the consequences of stress for the daily organization of behavior in a familiar environment where the animal (and the human) spends most of its time: the home cage (at home for humans).

3.2. Home cage observations

For patients that suffer from depression, the negative effects extend to both novel and familiar environments (Volkers et al. 2002; Keller et al. 2006). Animal models of depression have predominately assessed behavioral alterations in novel environments. In addition, the tests are short-lasting, and limited in the readout of the behavioral patterns. The few studies that address changes in circadian activity in mouse models include “chronic mild stress” and electric shocks which decreased the amplitude of circadian locomotor activity and food-intake (Willner 1984; Desan et al. 1988; Stewart et al. 1990; Gorka et al. 1996; Meerlo et al. 1999). To our knowledge, long-lasting analysis of activity patterns in the familiar environment of the home cage before, during and after a psychological stressor have not been described in mice.

Previous studies have shown that long term automatic recordings of the mouse in its home cage, allows detailed observations on dynamic changes in locomotor activity over days, with minimal human intervention (de Visser et al. 2005; de Visser et al. 2006) see Figure 3 for apparatus).

In addition, subtle changes in spontaneous behaviors under baseline conditions may reveal themselves more easily in the home cage than under conditions where the animal is prompted to explore or face a strong challenge. **Chapter 6** of this thesis will describe the daily organization of behavior in the familiar environment of the mouse’s home cage before, during and after chronic ’rat stress’.

Figure 3

We studied the impact of our chronic stress paradigm on undisturbed and novelty induced emotional, behavioral, cognitive and neuroendocrine responses that could underlie the expression of anhedonia in mice (**Chapters 6** and **7**).

3.3. How to measure anhedonia in an animal model?

To measure a diminished interest or responsiveness to positive stimuli, several methodological tools are available. In this thesis, we will use three tools: the sucrose consumption and preference test (**Chapters 6** and **7**), analysis of exploration patterns (**Chapter 6, 7** and **8**), and reward modulating effects on memory (**Chapters 5** and **7**).

An alteration in reward sensitivity can be measured using the sucrose task, where the consumption and preference for a sweet solution is determined. Depending on the experimental design, rodents are food and/or water deprived before testing takes place. However, as deprivation can induce stress, our experimental design did not use deprivation. Instead, during testing two bottles were presented for 24h. Allowing ample time for the mice to get 'used' to this new situation and drink when preferred. One bottle contains water, the other contains a sweet solution (5% sucrose: see Pothion et al. 2004); the bottles are weighed before and after a fixed time. The weight difference is a measure for fluid intake of both solutions, and the preference for either solution is calculated.

Novel stimuli may signal danger, but also possible rewards. During novelty exploration, activation of the avoidance-approach system occurs. Depending on the dominance of either system, exploration of the environment will be more or less intense. The hippocampus detects novel stimuli and is critical for the memory formation

of the novel event or environment. The novelty signal is also a major input to the brain reward mechanism, involving the neurotransmitter dopamine (Wittmann et al. 2007). Human fMRI studies show that joint activation of hippocampus and brain reward regions is crucial for the development of long term memories (Schott et al. 2006b). Thus, exploration patterns of a novel environment might provide leads to the emotional state of the animal (File 2001; Kalueff et al. 2006). Exploration is considered as self-rewarding behavior, involving the expectation of potential rewards, e.g., food, mates, a hiding place. While the inhibition of exploration is generally related to anxiety, it might also indicate the loss of hedonic responses, as suggested by Bevins and colleagues (Bevins and Besheer 2005).

Reward has been shown to affect the strength of memory (Huston and Mondadori 1977; Huston and Oitzl 1989; Messier 2004). We aimed to demonstrate that post-training access to sugar (the reward) will facilitate spatial memory of mice. This experimental set-up might allow to study whether exposure of mice to the chronic stress paradigm changes the perception of the emotional quality of the stimulus. The performance in the learning and memory task could reflect anhedonia. Consequently, we expect the loss of the memory facilitating effect of post-trial sugar administration in stressed mice.

The three tools that can measure the expression of anhedonia are part of the design of the experiments described in this thesis. We believe that multiple read-outs for loss of interest or pleasure, will underline the strength of our chronic stress model to induce anhedonia, and its relevance as an animal model of depression, a stress-related mood disorder.

4. Scope and outline of the thesis

4.1. Rationale and objectives

Chronic stress, defined as a hyper- or hypoactivity of the stress-system, in concordance with alterations in neuroendocrine-, emotional- and cognitive responses, are characteristics described for mood disorders like depression. To mimic these characteristics animal models are widely used. The overall aim of this thesis is to work towards a mouse model that expresses a wide range of signs and symptoms as seen with patients that suffer from depression, with special focus on the processes that underlie the expression of anhedonia.

The specific **aims** of the studies described in this thesis address methodological issues, home cage observations, activity patterns, emotional-, and learning and memory processes, with the objective to achieve translation of chronic stress effects in mice to humans by:

(i) Determining the circadian pattern of HPA axis activity and its molecular markers in the brain of naïve (non-stressed) mice at different ages.

(ii) Development of a stress-free method for oral drug delivery in mice, which allows to more specifically study the effect of the drug under study (i.e., CORT or mifepristone).

(iii) Characterization of learning and memory processes of mice in two distinct spatial learning tasks. The possibilities of either two tasks to measure a wide range of processes, will determine which spatial task will be used during subsequent behavioral testing.

(iv) Assessment of recurrent glucocorticoid receptor (GR) blockade effects on stress-system activity and behavior in novel environments, in naive mice.

(v) Characterization of the chronic 'rat stress' model by assessment of the neuroendocrine and behavioral responses in novel environments, i.e. learning tasks. In addition, investigation of the daily organization of behavior in the familiar environment of the home cage, before, during and following chronic 'rat stress'. The results will indicate whether anhedonia is expressed in our chronic stress model of depression.

(vi) Assessment of learning and memory in mice and humans with a history of chronic stress: translational study.

4.2. Experimental approach and outline

The experiments that are conducted can be divided in three categories addressing:

1. Methodological optimization: To design new, and optimize existing neuroendocrine and behavioral measurements to closely control the activation of the stress system, induced by the experimental procedures. Since timing, context and duration of a stressor determine the outcome of the experiments, interference by unintentional stressors has to be controlled and minimized (**Chapters 3** and **4**).

2. Longitudinal measurements: Home cage observations and novelty exposure are used to measure circadian behavioral and neuroendocrine activity patterns, as well as emotional responses and learning and memory performance. These measurements are combined with tests of anhedonia (**Chapters 2, 5, 6** and **7**).

3. Translational approach: Humans and mice that experience a period of chronic stress are subjected to comparable experimental designs which allow to test the use of distinct memory systems between the two species (**Chapter 8**).

For all experiments described in this thesis male mice from the C57BL/6J strain were used. There is abundant knowledge on the phenotype of C57BL/6J mice. Less is known about the circadian stress system activity in undisturbed conditions. In **Chapter 2**, the circadian activity of several HPA axis markers, with special focus on the 24h secretion pattern of CORT, is described for mice aged 3, 9 and 16 months. The results will be used as reference for comparison with the expected impact of chronic stress on circadian HPA axis activity (**Chapters 6** and **7**).

Next, we aim to optimize methodological procedures related to drug delivery and behavioral testing. Stress and CORT are known to affect memory processes. Experimental manipulation of mice, such as an injection, already induces stress-system activation, which most likely interferes with neuroendocrine and behavioral testing. In **Chapter 3** we set out to develop a non-invasive, stress free method of drug delivery via oats in mice. We will measure CORT in blood plasma in response to conventional drug delivery methods (*intraperitoneal* i.p., *per os* p.o.) and drug-delivery via oats. The latter method will allow close-context delivery of corticosteroids (and other drugs) prior to and directly after behavioral testing. Also, administration of CORT to mice in the undisturbed environment of the home cage, allows to study the effect of GR blockade on circadian HPA axis and behavioral activity (**Chapters 4** and **6**). We will investigate the effects of

single and repeated GR blockade (using RU38486/mifepristone) on circadian CORT patterns and behavioral responses in **Chapter 4.**

Learning and memory abilities can be assessed using a behavioral task. The choice of the task is based on task-inherent appetitive and aversive characteristics, amongst others. In **Chapter 5** we compare the behavior of mice tested in two spatial learning tasks that were originally designed for rats: the Morris water maze and the circular hole board (dry-land maze). Depending on the variability of the parameters that can be assessed by either behavioral task, further experiments described in this thesis would make use of one of the two spatial learning tasks to assess the impact of chronic stress on the novelty exposure and learning and memory processes (**Chapters 6, 7** and **8**). Memory can be modulated by positive and negative reinforcers delivered in close-context to the learning task. We will provide naïve mice with post-trial free access to sugar as positive reinforcer. This proof of concept is further used to determine whether mice exposed to our chronic stress paradigm display an alteration in learning and memory modulation by a reward (**Chapters 7** and **8**).

The effects of chronic stress in animal models are mainly assessed in short-lasting test-situations that have task-inherent features of novelty and sometimes even include exposure to a physical stressor. The experimental design described in **Chapter 6** is aimed to monitor in a longitudinal set-up, the daily organization of behavior in the familiar environment of the home cage before, during and following exposure to our chronic stress paradigm. Mice are repeatedly, and during unpredictable and uncontrollable times exposed to rats (without physical contact). In addition, we will test changes in the consumption of and preference for a sweet solution (sucrose = dissolved table sugar). The result could be indicative for changes in the reward system: loss of interest in pleasurable activities or diminished response to positive stimuli, also known as anhedonia.

In **Chapter 7** we combine methodologies as described in the previous chapters to reveal the effects of chronic 'rat stress' on learning and memory assessed in the circular hole board, and the memory modulating effects of a post-trial positive reinforcer. In addition, sucrose consumption and preference, exploration patterns during novel environment exposure, behavior in the light/dark box, and the pattern of CORT secretion is measured on several days after cessation of the stressor. The results from **Chapters 6** and **7** will additionally (together with **Chapter 5**) indicate whether our chronic stress paradigm induces the expression of anhedonia.

Multiple memory systems guide behavior. Acute stress modulates the contribution of memory systems to behavior in favor of caudate nucleus-dependent

stimulus response learning and memory, at the expense of hippocampus-dependent spatial learning and memory. In **Chapter 8** we examined whether chronic stress has similar consequences in mice and humans on the use of memory systems, as described for acute stress. The circular hole board task was modified to mimic the characteristics of the human task, allowing stimulus-response as well as spatial learning.

A general discussion of the findings is presented in **Chapter 9**, followed by a summary in **Chapter 10**.

Chapter 2

Age related changes in hypothalamic-pituitary-adrenal axis activity of male C57BL/6J mice

Sergiu Dalm
Leo Enthoven
Onno C. Meijer
Maaike H. van der Mark
Adriaan M. Karssen
E. Ron de Kloet
Melly S. Oitzl

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Abstract

As there is little known about age related changes in the hypothalamic-pituitary-adrenal (HPA) axis of mice, we determined the daily patterns of corticosterone secretion every 2h, together with adrenocorticotrophic hormone (ACTH) release and central HPA axis markers in the morning and evening in 3, 9 and 16 months old male C57BL/6J mice.

We observed that: (i) corticosterone secretion showed a distinct age related circadian pattern. During the light period this was expressed by relative hypercorticism in 9 months old mice and relative hypocorticism in 16 months old mice. ACTH was elevated at 16 months of age; (ii) mineralocorticoid- (MR) and glucocorticoid receptor (GR) mRNA expression in the hippocampus were significantly decreased in 9 months old mice, whereas in 16 months old mice, expression was similar to young animals. Circadian variation was modest in all age groups; (iii) the parvocellular hypothalamic paraventricular nucleus (PVN) expressed very high vasopressin mRNA, which was subject to circadian variation in 3 and 9 months-old mice. Furthermore, significant levels of MR mRNA were expressed in PVN.

In conclusion, basal HPA axis activity and expression of its central regulatory markers are age dependent in mice. This suggests that the capacity to adjust to environmental demands is either a function of age, or depends on different dynamics of the HPA axis.

Introduction

Corticosteroid hormones are potent modulators of neuronal functions. Circulating concentrations of cortisol and corticosterone are regulated by the Hypothalamic-Pituitary-Adrenal (HPA) axis, with argenine-vasopressin (AVP) and corticotrophin-releasing hormone (CRH) as the two main driving hormones from the hypothalamus (Dallman 2000). The central sensors of HPA axis activity in the regulation of feedback and other functions are embodied by the mineralocorticoid- and glucocorticoid receptor (MR and GR), which are expressed in discrete brain regions and in pituitary corticotrophs (Reul and de Kloet 1985).

The secretion of corticosteroid hormones exhibits a circadian pattern and can be induced by stressors (Akana et al. 1986; De Kloet et al. 1998; Windle et al. 1998b). These modes of corticosteroid secretion are regulated by inputs from the suprachiasmatic nucleus (Buijs et al. 1993), prefrontal cortex, amygdala and hippocampus, among others (Spencer et al. 1993). The latter region is an important target for corticosteroid hormones because it expresses high amounts of both MR and GR (De Kloet et al. 1998). Dramatic changes in circadian patterns of corticosteroid hormones have been described in aging and psychiatric diseases like depression and Alzheimer's disease (Hatfield et al. 2004; Peeters et al. 2004). The excessive activity of the HPA axis is generally associated with impaired mental and physical health (Sapolsky 1999; Lupien and Wan 2004) and characterized by increased basal and/or stress-induced levels of corticosteroid hormones and adrenocorticotrophic hormone (ACTH; Van Eekelen et al. 1995; Herman et al. 2001). Although numerous mouse models for a wide range of human stress related disorders have been developed, surprisingly little is known about the impact of age on basal regulation subserving circadian activity of the HPA axis in mice.

In the rat it is known that adjustments of the HPA axis occur in the course of life, that include changes in MR and/or GR protein and mRNA expression (Cai and Wise 1996; Bizon et al. 2001), as well as changes in secretagogue expression, and in adrenal sensitivity to ACTH. Accordingly, we expect to detect also in mice alterations in HPA axis activity that relate to normal life history. These changes may show individual and strain-specific differences as has been reported previously (Bazhanova et al. 2000; Workel et al. 2001).

In this study we have focused on the age dependent changes in circadian HPA axis activity of male C57BL/6J mice, aged 3-, 9- and 16 months. C57BL/6J is the most commonly used inbred mouse strain in research, and the preferential background strain for transgenic mouse models. We estimated corticosterone in blood plasma every 2 hours

to increase the likelihood to detect shifts or irregularities in the circadian pattern. At time points that were expected to coincide with circadian trough and peak concentrations of corticosterone in these mice (Grootendorst et al. 2004), we assessed plasma ACTH concentrations as well as mRNA expression of MR and GR in the hippocampus, and of MR, GR, CRH and AVP in the paraventricular nucleus (PVN) of the hypothalamus.

Materials and Methods

Animals

Male C57BL/6J mice were purchased from Janvier France at the age of 8 weeks. Upon arrival, mice were housed in groups of 8 mice per cage under SPF conditions (TNO, Leiden, The Netherlands). At the age of 3, 9 and 16 months (n = 16/group) they were transported to the animals facilities of the Sylvius Laboratories (Leiden, The Netherlands), acclimatized in a temperature (21 ± 1°C) and humidity (55 ± 5%) controlled room for two weeks. We chose 16 months as oldest age group, as it was reported that thereafter the survival rate might decrease (Talan and Ingram 1986). All groups were studied at the same time and at the same location, ruling out any differential environmental stimuli at the time of testing. Access to food and water was *ad libitum*; lights were on from 0700 - 1900h (12-12h light-dark cycle). To minimize HPA axis activation, mice were single housed from one day before blood sampling until the end of the experiment. They were also repeatedly handled. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

The circadian secretion of corticosterone was determined in blood samples collected via tail-incision every 2 hours for 24 hours. All age groups were divided in three sub-groups of 5 to 6 animals. As previously described (Durschlag et al. 1996), a small incision at the base of the tail with a razor blade allowed the collection of 50µl blood, within 90s after opening of the animal's cage. From each mouse, one blood sample was taken every 6 hours. Thus, each time point (with a 2 hour interval) consisted of 5 to 6 mice per group. During the dark period, blood sampling took place under red light conditions.

One week later, mice of each age group were distributed randomly over two groups: decapitation in the morning, 2 hours after lights turned on (0900h) and in the

evening, 2 hours before lights turned off (1700h). Decapitation took place within 15s of opening the animal's cage. Plasma concentrations of corticosterone were determined again as well as plasma ACTH. In the brains we determined the expression levels of molecular markers of HPA axis activity (n = 8 mice/time/age). Brains were snap frozen in isopentane pre-cooled on dry ice/ethanol and stored at –80°C until further use.

Hormone assays

Blood obtained via tail sampling and decapitation was collected individually in capillaries (coated with potassium-EDTA, Sarstedt, Germany), stored on ice and centrifuged with 13000 rpm at 4°C for 10 min. Blood plasma was stored at –20°C. Plasma corticosterone and ACTH concentrations were determined (in 10µl and 100µl plasma respectively) using commercially available radio immunoassay kits with ^{125}I-corticosterone and ^{125}I-ACTH (MP Biomedicals Inc., CA; USA; sensitivity 3ng/ml and 10pg/ml, respectively).

In situ hybridization

Brains were sectioned at –20°C in a cryostat microtome at 10µm in the coronal plane through the level of the olfactory bulb, piriform cortex, hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine coated slides (0.001%), air dried and kept at –80°C until further use.

In situ hybridizations using ^{35}S-labeled ribonucleotide probes (MR, GR, CRH, AVP) were performed as described previously (Schmidt et al. 2003). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense RNA probes were transcribed from linearised plasmids containing exon 2 of mouse MR and GR, the full length coding regions of CRH (rat) and exon C of the rat AVP gene (with 92% homology to mouse). Tissue sections (3 – 4 / slide) were saturated with 100µl hybridization buffer containing 20mM Tris-HCl (pH 7.4), 50% formamide, 300mM NaCl, 1mM EDTA (pH 8.0), 1x Denhardt's, 250µg/ml yeast transfer RNA, 250µl/ml total RNA, 10mg/ml salmon sperm DNA, 10% dextran sulfate, 100mM dithiothreitol, 0.1% SDS, 0.1% sodium thiosulfate and supplemented with approximately 1.5 x 10^6 cpm ^{35}S-labeled riboprobe. Brain sections were cover slipped and incubated overnight at 55°C. The next day sections were rinsed in 2 x SSC, treated with RNaseA (20mg/ml) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1 x SSC at 65°C for 30 min and dehydrated through increasing concentrations of ethanol. All age groups were assayed

together. Films were apposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) and developed. For AVP, slides were dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, NY) and exposed at 4°C for 5 days. Slides were developed, counterstained with Toluidine Blue and examined with a light microscope using both bright and dark field condensers.

Autoradiographs were digitized, and optical density (O.D.) of the areas of interest was quantified using image analysis computer software (analySIS 3.1, Soft Imaging System GmbH). The average density of 4 - 8 measurements for each animal was calculated. For AVP measurement, the area of the parvocellular part of the PVN was determined by light microscopy and the number of radioactive labeled cells was counted.

Statistical analysis

Data are presented as mean ± S.E.M. The circadian profile of corticosterone was analyzed by analysis of variance (ANOVA; factor: age) with repeated measurements followed by LSD *post-hoc* test. Total corticosterone (AUC: area under the curve) over 24 hours, as well as separately for light and dark periods, were subjected to ANOVA, with age (3, 9 and 16 months) and time of the day (day and night) as fixed factors. Corticosterone and ACTH concentrations and mRNA expression of the various HPA markers collected in the morning and evening were analyzed by ANOVA with age and time of the day (morning, evening) as fixed factors. Significance was accepted at $p < 0.05$.

Results

Circadian rhythm of corticosterone

Mice of all ages showed a circadian rhythm of corticosterone (Figure 1; time $F_{(11,165)}=33.32$, $p < 0.05$) with age dependent characteristics (age $F_{(2,15)}=4.64$, $p < 0.05$). Corticosterone secretion increased from 1400h onwards in all groups with peak values at the end of the light phase and the beginning of the dark phase (between 1600 and 2200h). Interestingly, the course of the circadian rhythm was age dependent (age * time $F_{(22,165)}=3.78$, $p < 0.05$). Corticosterone of 3 months old mice was low during the early light phase (0800 - 1200h; ± 20ng/ml), increased during the late light phase (1800h) followed by a clear peak at 2000h (± 100ng/ml). Thereafter, corticosterone reached baseline resting levels (± 20ng/ml) within 2 hours and remained low during the remainder of the dark period.

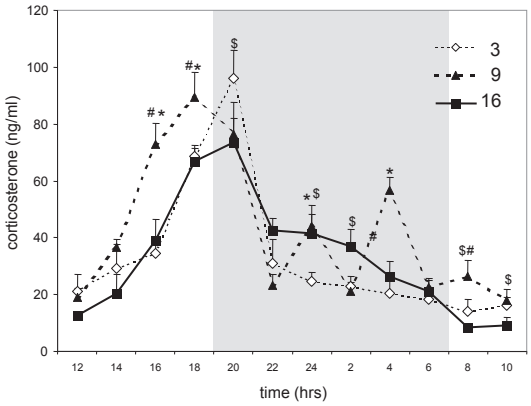

Figure 1
Circadian secretion of corticosterone in ng/ml (mean ± S.E.M.), measured every 2 hours in blood plasma of 3, 9 and 16 months old male C57BL/6J mice, entrained in a 12-12h light-dark cycle (dark phase from 1900 to 0700h represented by the gray shaded area). *P*-value < 0.05: * 3 vs. 9 months; $ 3 vs. 16 months; # 9 vs. 16 months.

The circadian profile of 9 months old mice showed elevated corticosterone already during the early light phase, reached peak corticosterone secretion earlier (at 1600h) than the other two age groups with a prolonged duration (1600 - 2000h). Although corticosterone returned to basal within 2 hours, irregular peaks of corticosterone were found during the dark and early light phase (2200 - 0800h).

The 16 months old mice had low corticosterone levels during the early light phase (0800 - 1200h; ± 10ng/ml), reaching peak concentrations from 1800 to 2000h (± 75ng/ml), however, it was at a lower level than the other two age groups. Although corticosterone had decreased at 2200h, it remained elevated during the following period (until 0400h) and dropped at the beginning of the light period to levels below the other two age groups.

Corticosterone: total amount

The total amount of corticosterone changed with age (AUC: age $F_{(2,18)}$=8.93, p = 0.003). AUC-values over 24 hours were significantly increased in 9 months old mice (58.0 ± 2.0 x 10^3) compared to 3 (49.2 ± 2.8 x 10^3; p < 0.01) and 16 months old mice (45.8 ± 1.3 x 10^3; p < 0.001). Separate calculations for the light and dark phase revealed for the dark phase an age-independent corticosterone AUC value. However, age groups differed during the light phase ($F_{(2,18)}$=36.45, p < 0.0001): corticosterone was lowest for the 16 months old mice (19.3 ± 0.9 x 10^3), highest at 9 months (31.0 ± 0.9 x 10^3) and intermediate at 3 months of age (25.4 ± 1.1 x 10^3) with significant differences between all groups (p < 0.001).

HPA axis activity in the morning and evening

To gain more insight in the regulation of the HPA axis we decapitated the animals at two time points during the day and harvested blood and brain for the detection of hormones and expression of selected mRNAs.

Corticosterone and ACTH

Plasma corticosterone and ACTH concentrations (Table 1) were measured in blood samples, collected by decapitation in the morning (0900h) and towards the evening (1700h). Morning corticosterone was age dependent ($F_{(2,50)}$=12.77, $p < 0.001$) as it was significantly lower in 16 months old mice ($p < 0.05$ vs. 3 and 9 months). All age groups showed increased evening corticosterone ($F_{(2,24)}$=8.03, $p < 0.002$), which was highest at 9 months of age ($p < 0.05$ vs. 3 and 16 months), confirming the circadian measurements. For ACTH, morning and evening values were comparable, but affected by age ($F_{(2,42)}$=13.06, $p < 0.001$); while ACTH was similar in 3 and 9 months old mice, it was increased at 16 months of age at both times ($p < 0.05$; vs. 3 and 9 months).

Table 1:
Basal morning and evening concentrations of corticosterone (ng/ml), ACTH (pg/ml), and expressions of MR, GR, CRH mRNA in the paraventricular nucleus (PVN) of the hypothalamus (arbitrary units of optical density) in 3, 9 and 16 months old C57BL/6J mice.

	Morning			Evening		
	3	9	16	3	9	16
Corticosterone	16.1 ± 2.2	20.5 ± 1.0	**9.0 ± 0.8** $^{\#\,\$}$	52.2 ± 4.6†	**84.7 ± 8.6**$^{*\,\#\,\dagger}$	55.4 ± 4.4 †
ACTH	63.7 ± 8.2	76.4 ± 14.9	**156.8 ± 26.6**$^{\#\$}$	63.0 ± 3.0	79.1 ± 14.3	**134.8 ± 15.7** $^{\#\,\$}$
MR	21.7 ± 0.7	17.8 ± 2.1	16.9 ± 1.4	**25.1 ± 1.5**$^{+}$	**22.5 ± 2.1**$^{+}$	**20.7 ± 1.4**$^{+}$
GR	61.8 ± 5.8	57.0 ± 5.8	57.5 ± 3.2	65.7 ± 5.5	54.4 ± 2.8	58.4 ± 9.7
CRH	27.2 ± 4.2	31.1 ± 2.1	27.2 ± 3.9	24.0 ± 6.2	33.8 ± 4.4	29.3 ± 4.8

Bold numbers indicate statistically significant differences. Data are presented as mean ± S.E.M.
P-value < 0.05: * 3 vs. 9 months; $^{\#}$ 9 vs. 16 months; $^{\$}$ 3 vs. 16 months; $^{+}$ morning vs. evening

MR and GR mRNA expression in the hippocampus

Overall hippocampal MR mRNA expression was differentially affected by age and time (Figure 2; age $F_{(8,60)}$=3.44, p = 0.003; time $F_{(4,30)}$=12.16, p = 0.001). Differences between

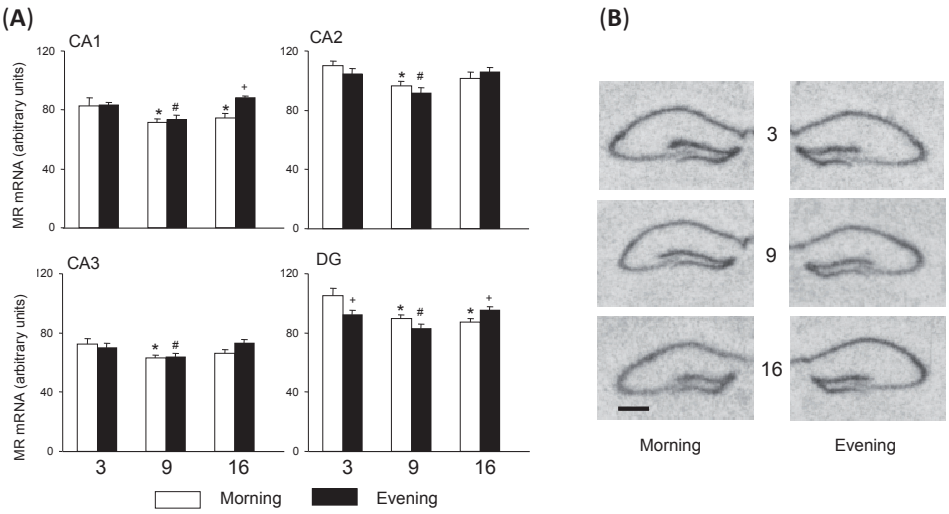

Figure 2

Expression of MR mRNA in the hippocampal subfields CA1, CA2, CA3 and dentate gyrus (DG) of 3, 9 and 16 months old C57BL/6J mice, in the morning and evening hours. **(A)** mean ± S.E.M. **(B)** Representative photomicrographs; bar = 1 mm; bregma –1.70 mm. *P*-value < 0.05: * vs. 3 months; # 9 vs. 3 and 16 months; + morning vs. evening.

groups were in the range of 10 to 15%. The age effect was significant for all subfields (CA1: $F_{(2,33)}$=7.755, p = 0.002; CA2: $F_{(2,33)}$=7.17, p = 0.003; CA3: $F_{(2,33)}$=5.42, p = 0.009; DG: $F_{(2,33)}$=9.21, p = 0.001). In the morning, 3 months old mice had the highest MR mRNA expression (3 vs. 9 months: all subfields p < 0.05; 3 vs. 16 months: DG - p < 0.05). Nine-months old mice had the lowest MR mRNA expression compared to the other two age groups. In the dentate gyrus (DG), we found an interaction between age and time (DG: $F_{(2,33)}$=6.07, p = 0.006): in the evening, MR mRNA was lower in 3 months old mice, but increased in 16 months old mice, with no change at 9 months of age. While MR mRNA showed no circadian variation in other subregions of the hippocampus in 3 and 9 months old mice, it was increased in the CA1 in the evening at 16 month of age.

Expression of GR mRNA was about 20 to 30% lower in 9 months old mice (Figure 3; age $F_{(6,66)}$=2.57, p = 0.027), compared to 16 months old mice in CA1, CA3 and DG (all p < 0.05). Expression of GR mRNA was similar at 3 and 16 months of age. Time of the day did not affect GR mRNA expression.

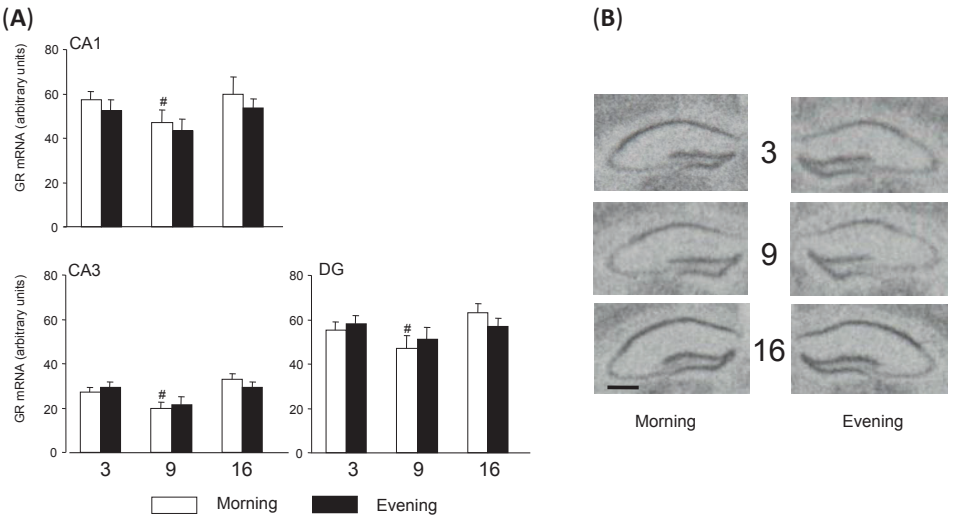

Figure 3

Expression of GR mRNA in the hippocampal subfields CA1, CA3 and dentate gyrus (DG) of 3, 9 and 16 months old C57BL/6J mice, in the morning and evening hours. **(A)** mean ± S.E.M. **(B)** Representative photomicrographs; bar = 1 mm; bregma –1.70 mm. *P*-value < 0.05: # 9 vs. 16 months.

MR, GR, CRH and AVP mRNA expression in the PVN of the hypothalamus

Expression of MR mRNA in the PVN was not affected by age, but was higher in the evening at all ages (time $F_{(1,32)}$=6.26, p = 0.02; Table 1). No differences in GR and CRH mRNA expression were found (Table 1).

The strong mango- as well as parvocellular paraventricular expression of AVP mRNA was not affected by age, but by time of the day (Figure 4; $F_{(1,43)}$=9.39, p = 0.004), in both the magno- ($F_{(1,41)}$=7.71, p = 0.009) and parvocellular part of the PVN ($F_{(1,41)}$=6.08, p = 0.022). In both subregions, AVP mRNA was lower in the evening than in the morning of 3 and 9 months old mice (p < 0.05), while it was comparable for 16 months old mice.

MR and GR mRNA expression in other brain areas

MR and GR mRNA are also expressed in other brain areas, which are not known to be involved in circadian HPA axis regulation. Therefore, we decided *post hoc* to screen the olfactory bulb for MR mRNA and the piriform cortex for both MR and GR mRNA expression, to detect age- and possible brain-site specific changes of MR and GR mRNA.

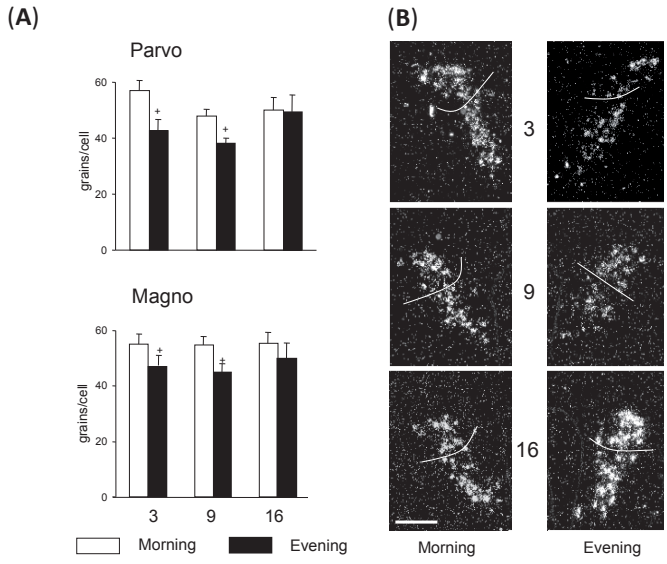

Figure 4

Expression of AVP mRNA in the magno- and parvocellular part of the paraventricular nucleus (PVN) of the hypothalamus of 3, 9 and 16 months old C57BL/6J mice, measured in the morning and evening. **(A)** mean ± S.E.M. grains/cell. **(B)** Darkfield photomicrographs of AVP mRNA in the morning (left) and evening (right); lines indicate partition between magno- and parvocellular part of the PVN; bar = 0.1 mm. Note the strong expression of AVP mRNA in both the magno- and parvocellular PVN. *P*-value < 0.05: $^{+}$ morning vs. evening.

No significant differences for age or time of the day were detected in any of these brain areas (data not shown).

Bodyweight

Bodyweight showed the expected age related increase ($F_{(2,49)}$=205.61, $p < 0.001$; in gram - mean ± S.E.M: 3 months 26.3 ± 0.2; 9 months 31.3 ± 0.4; 16 months 37.6 ± 0.3; $p <$ 0.001 between all groups).

Discussion

We have characterized several novel elements of HPA axis activity in 3, 9 and 16 months old male C57BL/6J mice during a circadian 12-12h light-dark cycle. Age associated changes notably consisted of shifts in time, amplitude and regularity of corticosterone secretion over time, and elevated ACTH levels in 16 months old mice only. Aging was also reflected in a clearly differential pattern of hippocampal MR and GR mRNA expression, with lowest GR mRNA expression present in the intermediate age group. In the PVN, no age related changes were detected for MR, GR, CRH and AVP mRNA, while MR and AVP mRNA expression showed circadian variation.

Age and circadian variations of hormones

High concentrations of cortisol and corticosterone, interpreted as hypercorticism are frequently reported in aged humans and rats, particularly when determined in blood samples collected during the active period (Meaney et al. 1992; Lupien and Wan 2004). In the present study, we found corticosterone to be highest for the 9 months old and lowest for the 16 months old mice during the light, inactive period with no differences in total AUC corticosterone over the dark, active period. However, if the time course during the active period is taken into account, the prolonged elevation of corticosterone after the peak indicates a transient relative excess of corticosterone at 16 months of age. At this age, a subsequent period of low corticosterone follows, resulting in a total daily exposure of corticosterone similar to that observed in 3 months old mice. These findings suggest that, particularly in the oldest group of mice the total daily exposure of the organism to corticosterone is tightly controlled.

Corticosterone secretion from the adrenals is stimulated by ACTH. Whereas corticosterone showed a clear circadian pattern, we were unable to detect circadian variation of ACTH during the light period. The preferred method of sampling for ACTH would be via intravenous cannulation, but even then, variations in ACTH during the light period have been reported to be marginal in rats (Atkinson and Waddell 1997; Watts et al. 2004). For practical reasons, we had to collect blood via decapitation, which also can be used to detect variations in ACTH (Bradbury et al. 1994; Watts et al. 2004). We guaranteed low basal HPA axis activation via single housing and repeated handling before the start of decapitation similar to Atkinson *et al.* (Atkinson and Waddell 1997). We previously observed that single housing is necessary to obtain low plasma ACTH measurements from decapitated mice (S. Dalm and O.C. Meijer; unpublished data). We

interpret the low variability of the ACTH levels in combination with the clear significant increase in 16 months old mice as an argument for the basal character of the ACTH levels.

The sensitivity of the adrenals to ACTH is part of the mechanism underlying the regulation of circadian corticosterone secretion (Akana et al. 1986). Adequate corticosteroid production is associated with successful aging in rats and humans (Workel et al. 2001; Lupien and Wan 2004), while excessive corticosteroids impair mental and physical health (Sapolsky 1999). In contrast to the reports on age dependent hypercorticism, numerous studies indicate an apparent reduction in the sensitivity of the adrenals to ACTH stimulation, concomitant with 'normocorticism' (Carnes et al. 1994; Van Eekelen et al. 1995; Magri et al. 1997; Workel et al. 2001). Increased morning and evening ACTH of 16 months old mice confirm those findings in humans and certain rat strains. The adrenals of 16 months old mice appear to be hyposensitive, since more ACTH is required to induce either lower or similar corticosterone secretion during the morning and evening compared to 3 months old mice. This suggests that in older animals a minimum corticosteroid level is maintained via an adaptation in the sensitivity of the adrenals towards ACTH. In contrast, at 9 months of age ACTH and corticosterone concentrations point to an adrenal hypersensitivity as was previously observed in the rat (Akana et al. 1986).

Central markers of HPA axis activity

The changes in hormone levels with age were accompanied by variations in central markers of HPA axis activity. We are aware that these mRNA markers provide only an estimate of functional changes across age and the circadian cycle without defining the physiological impact, but they do allow comparison with other studies in mouse and rat.

Like in rats (van Eekelen et al. 1991; Van Eekelen et al. 1995; De Kloet et al. 1998; Sapolsky 1999), hippocampal MR mRNA expression of mice modestly decreased with age but whereas in 9 months old mice expression was decreased by 10 - 15% both in the morning and evening, signal intensity was increased in 16 months old mice up to the level of 3 months old mice during the evening. Similar to young rats (Spencer et al. 1993; Holmes et al. 1997), MR mRNA was decreased in the dentate gyrus of 3 months old mice in the evening. In 9 months old mice, hippocampal GR mRNA expression was decreased by 20 - 30%. No circadian changes were observed. Apparently MR and GR mRNA expression in the hippocampus oscillates as a function of age, with more circadian changes at the level of MR.

Unexpectedly and in contrast to findings in rats (Cizza et al. 1995; Workel et al. 2001) the expression of MR and GR mRNA in the PVN was independent of age. Compared to the hippocampus, MR mRNA expression was lower in the PVN, but clearly detectable. It showed a pronounced circadian rhythm, with increased levels in the evening at all ages. This emphasizes that expression profiles of hippocampal corticosteroid receptor mRNA cannot be generalized to other brain structures nor are they predictive for age related alterations.

CRH is generally considered the principal neural signal controlling (stress-induced) ACTH release whereas AVP is considered to weakly stimulate ACTH release on its own but to markedly amplify the effect of CRH (Kalsbeek et al. 2002; Watts et al. 2004). Both CRH and AVP mRNA expression in the PVN did not change with age. Interestingly, AVP mRNA decreased in the evening in 3 and 9 months old mice but was constant over the day at 16 months of age, which also showed highest ACTH plasma levels. Elevated AVP has been reported in aged humans and rats (Zhou and Swaab 1999; Keck et al. 2000), but although our data may suggest elevated evening levels of AVP mRNA, ANOVA indicated no significant age effect. Novel, and in contrast to rats (Keck et al. 2000; Itoi et al. 2004), is the finding that AVP mRNA is expressed in similar amounts in both parvo- and magnocellular neurons of C57BL/6J mice. Up to now only certain conditions like adrenalectomy in rats are known to induce AVP mRNA in the parvocellular part of the PVN (Grillo et al. 1998; Itoi et al. 2004).

Corticosterone is a regulator of MR, GR (Spencer et al. 1993; Herman and Spencer 1998; Spencer et al. 1998) and CRH and AVP (Sawchenko 1987; Kovacs et al. 2000) mRNA expression. Reciprocally, MR mediates the action of corticosterone on basal HPA axis activity, while GR is mainly involved in the stress-related actions (Ratka et al. 1989; de Kloet et al. 1993b). The reduced hippocampal MR and GR mRNA, therefore, could be either consequence or (part of the) cause of the elevated and irregular secretion pattern of corticosterone in the 9 months old mice. Correlative studies in rat suggest that decreased hippocampal GR expression does not necessarily depend on elevated glucocorticoids, but might be a consequence of aging *per se* (Murphy et al. 2002).

Basal levels of CRH and AVP are under feedback inhibition by corticosterone (Ma and Aguilera 1999). The circadian rise of corticosterone might be associated with the decreased AVP mRNA of 3 and 9 months old mice in the evening. However, at 16 months of age, AVP mRNA remained high in the face of the increased evening corticosterone, together with elevated ACTH. Presumably, the elevated AVP mRNA amplifies the CRH effect on ACTH, thus subserving appropriate corticosterone production from a hyposensitive adrenal. Although the discussion of regulatory mechanisms is challenging

and could be elaborated, it remains speculative, as we did not attempt to perform experimental manipulations of the HPA axis.

Some features of the HPA axis showed remarkable changes during the life of C57BL/6J mice. However, the consequences of these changes in circadian and age dependent patterns of HPA axis activity for the regulation of the stress response and other brain functions like cognitive processes (de Kloet et al. 1999), remain to be elucidated. We and others have shown in rats that early life stress due to maternal deprivation changes HPA axis activity, stress reactivity and cognitive performance throughout life, underlining the importance of an undisturbed development of the HPA axis (Oitzl et al. 2000; Workel et al. 2001). Only if the HPA axis fails to adapt during the aging process, physiological and behavioral processes may be compromised (Everitt and Meites 1989; Meijer et al. 2005).

Concluding, measurement of the aging circadian HPA axis activity in the mouse reveals adaptations at various levels. It appears that there are oscillations in the activity of the various components of the HPA axis rather than linear progressive functional changes. Similar as has been shown in the aged rat, high ACTH was accompanied by low corticosterone secretion. We propose that the adaptive changes in adrenal sensitivity and brain corticosteroid receptor mRNA preserve homeostasis in corticosteroid exposure throughout life.

Acknowledgements

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Chapter 3

Non-invasive stress-free application of glucocorticoid ligands in mice

Sergiu Dalm
Vera Brinks
Maaike van der Mark
E. Ron de Kloet
Melly S. Oitzl

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Abstract

Most drug delivery procedures induce stress which might interfere with the pharmacological action of the drug and behavior. Stress is deduced from high and long-lasting elevations of the hormone corticosterone. We set out to develop a non-invasive, stress-free method of drug delivery in mice. Validation consisted of delivery of glucocorticoid ligands via oats to male C57BL/6J mice.

Oat consumption induced a small increase in corticosterone concentrations after 15 min (< 50ng/ml) that returned to low resting levels at t=30 (< 10ng/ml). Gavage and intraperitoneal vehicle injections resulted in long-lasting corticosterone elevations (> 100ng/ml at t=30 and ~ 50ng/ml at t=60 min after delivery). Adding corticosterone to oats resulted in 3-fold higher plasma corticosterone in the 15.0mg/kg-group (± 250ng/ml) compared to the 4.5mg/kg-group at t=30 and t=90. Application of the glucocorticoid receptor antagonist RU38486 (200mg/kg) elevated the plasma corticosterone levels for at least eight hours. Additional swimming increased corticosterone even further. Presumably, already the small oat-consumption-induced increase of corticosterone requires negative feedback via glucocorticoid receptors.

In conclusion, the context-dependent and dose-controlled application of drugs via oats avoids confounding strong stress system activation and makes it suitable for studies on learning and memory processes.

Introduction

Assessment of pharmacological drug profiles, but also studies on mechanisms underlying cognition and behavior, require the controlled application of drugs. Most procedures related to administration of drugs to small laboratory animals like mice, require invasive methods. Already hand-restraint will lead to a concomitant, non-controlled and unwanted activation of the stress system (Balcombe et al. 2004). By definition, any kind of stress, even a mild stressor, is a potential confounding factor of drug effects. Specifically, in relation to the well known effects of stress on cognitive processes (Lupien and McEwen 1997; Joels et al. 2006) a non-invasive, stress-free and dose- and time-controllable drug-delivery is of crucial importance, but often disregarded or discarded as neglible.

The stress response in humans and rodents is controlled by the Hypothalamic-Pituitary-Adrenal (HPA) axis. In mice and rats, corticosterone is the glucocorticoid hormone secreted from the adrenals in response to stress, i.e., any event that disturbs the psychological and physiological homeostasis of the organism. The effects of corticosterone are mediated in the brain by two nuclear receptors: the high affinity mineralocorticoid receptor (MR) and the low affinity glucocorticoid receptor (GR). Neuroendocrine regulation via MR controls basal HPA axis activity and sensitivity to a stressor. GR is activated after high circulating corticosterone levels, exerting negative feedback and facilitation of the essential recovery from the stress response (De Kloet et al. 1998). Measurement of circulating plasma corticosterone concentrations is an accepted tool to assess stress-induced activation of the HPA axis.

Drug-delivery via food or drinking water is an easy to perform, non-invasive procedure for mice, however lacking dose- and time-controlled deliveries (Ruzek et al. 1999). Although the route of administration (*per os*) fits a stress-free application form, dose- and time-control has to be accomplished differently. Therefore, we address the potential of using a treat or bait to deliver drugs. This has previously been shown effective in other species like birds, where mealworms injected with corticosterone were supplied in close context with the requested behavioral response (Breuner et al. 1998). As a treat, we decided to use oats as mice like to eat them and the structure of oats facilitates the soak up of solutions. To validate the method, we administered glucocorticosteroids ligands to manipulate HPA axis activity and subsequently measured blood plasma corticosterone concentrations.

The aim of the experiments was to devise a non-invasive stress-free, dose- and time-controlled procedure for effective delivery of glucocorticoid agonists and -antagonists to mice. In context with the procedures of drug administration, corticosterone

concentrations were measured in blood plasma at various time points. First, we tested the hypothesis that mice would readily consume the oats, without concomitant increase in endogenous corticosterone levels. Vehicle applied via a gavage or intraperitoneal injection was expected to result in higher corticosterone concentrations. Second, we investigated the dose- and time-dependency of corticosterone treatment in oats. Finally, the effect of the GR antagonist RU38486 was determined.

Materials and Methods

Animals and housing

Ten weeks old male C57BL/6J mice were purchased from Janvier Bioservices (Netherlands). Upon arrival, the mice were single housed with food and water *ad libitum* and allowed to acclimatize for two weeks to the testing room. The room was temperature (19 – 21 °C) and humidity (50 - 60%) controlled; lights on from 0700 to 1900h (12-12h light-dark cycle). Animals were repeatedly handled, weighed and tested between 0900 and 1400h. The experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Familiarization to oat administration

One week prior to the start of the experiments, a feeding-cup (2.3cm diameter x 2.5cm high; Figure 1A) was glued to the floor in a corner of the home cage, opposite the nest location. For familiarization, three flakes of oats (Speltvlokken, Biologische teelt, Graanpletterij de Halm, Netherlands; ± 140mg) were placed in the cup on three consecutive days, every other day at 0900h. The grid of the cage was lifted and the sawdust was removed from the cup using an air puff generated with a pipette. Next, the oats were placed into the cup using forceps to minimize human odor transfer. Thereafter, the cage was closed and the mouse was allowed to eat the oats undisturbed. All the oats were consumed within 10 min (Figure 1B/C).

Drugs

Oat delivery: One day prior to the experiment, three flakes of oats were placed in a glass vial and the solutions containing corticosterone, GR antagonist or dissolvent were applied. The glass vials containing the oats were kept at room temperature over

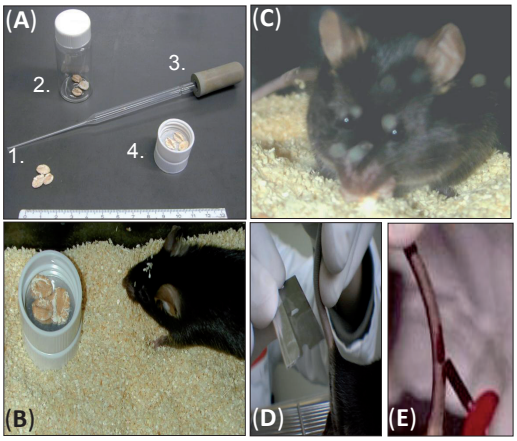

Figure 1
Tools for drug administration via oats and subsequent blood sampling via tail incision. **(A)** prerequisites used during preparation: 1) oats, 2) glass vial, 3) pipette, 4) feeding cup; and ruler giving the size of the objects in cm; **(B)** three flakes of oats are placed with forceps in a feeding cup in the home cage of the mouse; **(C)** mouse eats oats; **(D)** incision of the tail with a razor blade to allow **(E)** blood sampling.

night. Within 16 hours, the solution was absorbed by the oats and they were dry when presented to the mice.

Corticosterone was dissolved in CORT-HBC complex (Sigma-Aldrich Cole; Germany) in 5.6 and 18.7ng/ml and 0.9% NaCl-HBC as vehicle (VEH). From these solutions 20µl was applied to three oats resulting in a corticosterone dose of 4.5 and 15.0mg/kg for the treatment groups (Ruzek et al. 1999).

The GR antagonist RU38486 (100mg/ml; kindly provided by Corcept Pharmaceuticals, CA, U.S.A.) was dissolved in 1ml 0.9% NaCl containing 0.25% carboxymethylcellulose and 0.2% Tween20 (VEH). From this solution 50µl was applied to the oats (mice received 200mg/kg RU38486).

Gavage injection: The mouse was hand restraint and a gastric feeding needle (length = 3cm; 19 gauge = 1mm diameter; BioService, Belgium) was used to apply a volume of 200µl/25g bodyweight *per os.*

Intraperitoneal (i.p.) injection: The mouse was hand restraint and kept on its back within the palm of the hand. The injection (needle: 25 gauge = 0.5mm) was given in the lateral aspect of the lower left quadrant of the belly.

Adrenalectomy

To start surgery with low stress system activity, mice were transported to the operation room two hours earlier. Between 0900 to 1400h adrenalectomy (ADX) was performed using the dorsal approach under isoflurane anesthesia. The percentage of isoflurane used to induce anesthesia was 4%, and was decreased to 2% at the moment of surgery. The adrenals were removed and surgery was completed within 5 min. Mice received

two bottles, one containing 0.9% saline, and the other containing water, upon return to their home cage.

Blood sampling and corticosterone assay

Corticosterone concentrations were measured in blood samples obtained via tail incision (Figure 1D/E). Briefly, a small incision with a razor blade at the base of the tail allowed collection of < 50µl blood within 60s after opening of the animal's cage (Durschlag et al. 1996; Dalm et al. 2005). We refined the method to minimize possible confounding factors of the incision procedures and concomitant blood volume reduction (Grassler et al. 1990). The mouse is only touched at the tail, not fixated in the hand (Figure 1D/E). Only a minute volume of blood was collected, i.e., < 50µl, with at least 60 min between two blood samples. To stop bleeding, the tail was gently pressed into the sawdust of the cage. Separate groups of mice are used for the time-course measurements. The entire procedure lasted less than 60s. Data on Oats+VEH treated mice in Figure 2 support the efficacy of our blood sampling method. We conclude that the blood sampling procedure did not significantly contribute to the observed pharmacological effects of our experimental design.

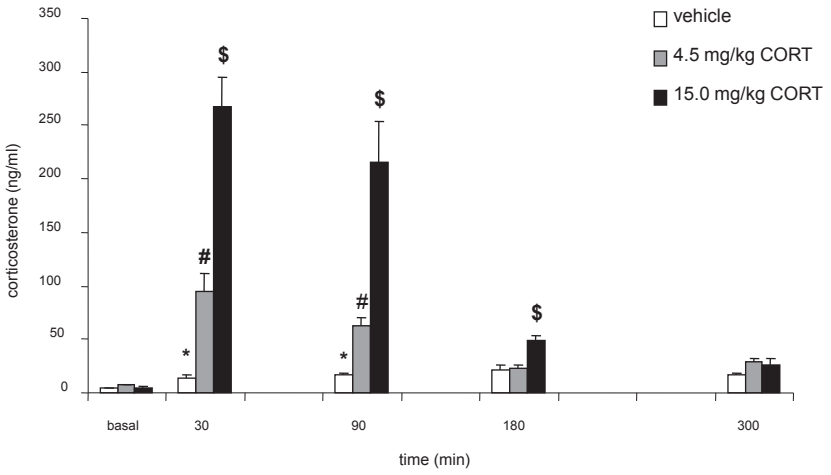

Figure 2

Dose-controlled delivery of corticosterone (CORT: 0, 4.5 and 15.0mg/kg bodyweight) via oats (indicated by arrow at 0900h) to adrenalectomized mice. Plasma corticosterone concentrations in ng/ml on the day before (basal), and t=30, 90, 180 and 300 min after oat consumption. Note that all corticosterone values of ADX mice are in the range of basal corticosterone secretion. Data are presented as mean ± S.E.M.; $p < 0.05$: * vs. other groups.

At the end of the experiments, blood samples were taken from trunk blood. Blood was collected individually in capillaries, coated with potassium-EDTA (Sarstedt, Germany), stored on ice and centrifuged with 13000 rpm at 4°C for 10 min. Plasma was stored at –20°C. Corticosterone was analyzed using a commercially available radio immunoassay kit ^{125}I-corticosterone (MP Biomedicals, Inc., NY, USA; sensitivity 3ng/ml).

Blood glucose levels in response to oat consumption

In response to food consumption the blood glucose levels rise (Gagliardino et al. 1984). Basal glucose levels were determined one day before three flakes of oats (± 140mg) were presented in the feeding cup of the mice (two groups of mice (n = 8/group). Blood samples were taken after 5, 15 and 60 min after oat presentation (group 1) and after 10 and 20 min (group 2). A droplet of blood was applied to a test strip and within seconds the glucose content of the blood was displayed in mmol/l (Accu-Chek Compact, Roche Diagnostics, GmbH, Mannheim, Germany). Then, the mouse was returned to its home cage (total duration < 15s).

Experiment 1: Methods of drug-delivery and HPA axis activity

To determine whether and to what extent the methods of drug-delivery (including moving the cage, eating oats, the vehicle, gavage and intraperitoneal injections) affect HPA axis activity, we estimated corticosterone concentrations at multiple time points.

Oats procedure: One day before the experiment started, blood samples were taken to determine basal corticosterone concentrations. Mice were distributed randomly to three groups (n = 10 – 11/group): (1) Delivery control procedures: get the cage from the shelf, lift grid, touch feeding cup with forceps, close cage and return it to the shelf; (2) Consumption of pure oats; (3) Response to vehicle: consumption of oats with absorbed dissolvent used for the GR antagonist RU38486. Blood was collected from the same mice either at 30 or 60 min after the oat procedures on two consecutive days.

Gavage injection*:* Two groups of mice (n = 8/group) were injected *per os* with 200µl VEH (see above).

Intraperitoneal injection: Two groups of mice (n = 8/group) were injected intraperitoneal with 200µl VEH.

Handling procedures related to gavage and intraperitoneal injections (mice, n = 8): get the cage from the shelf, lift grid, pick up the mouse from the cage at the base of the tail, place mouse on grid, restrain the mouse as preparation for either gavage and intraperitoneal injection for 5s, return mouse to cage.

Experiment 2: Dose-controlled corticosterone administration via oats

To differentiate the amount of exogenously administered corticosterone in the blood from the endogenously secreted hormone, mice were adrenalectomised (ADX). In contrast to rats, ADX-mice remain to secrete basal low concentrations of corticosterone from scattered cell groups in the vicinity of the adrenals (Hummel 1958). ADX mice lack the stress- or circadian-induced increase in corticosterone secretion, keeping a basal secretion of corticosterone between 5 and 25ng/ml. To verify the quality of ADX, basal blood samples were taken in the evening five days after surgery. Two days later at 0900h, mice (n = 8/group) received three flakes of oats containing 4.5 or 15.0mg/kg corticosterone or vehicle. Blood samples were taken at t=30, 90, 180 and 300 min following oat consumption.

Experiment 3: GR antagonist RU38486 delivery via oats

Activation of GR regulates the negative feedback on corticosterone secretion during the circadian peak and in response to stress (De Kloet et al. 1998). Blockade of the GR inhibits negative feedback. Consequently, corticosterone concentrations increase or remain elevated. Mice ate oats with the GR antagonist RU38486 or vehicle (factor: treatment). One hour later, half of the mice had to swim in a bucket filled with warm water (30cm diameter x 40cm high; 26 ± 1°C) for 1 minute, to activate the HPA axis (factor: condition). The mouse was removed from the water using a grid and returned to its home cage which was placed underneath a heating lamp (250Watt) for 3 min. Control mice remained in their home cage. We hypothesized that blockade of GR by RU38486 would result in high concentrations of corticosterone in mice exposed to swim stress.

According to treatment and condition, four groups were formed (n = 8/group): (1) Oats + RU38486 + swim; (2) Oats + VEH + swim; (3) Oats + RU38486 + no-swim; (4) Oats + VEH + no-swim. Basal corticosterone concentration was determined between 0900 to 1000h, one day before the start of the experiment. The next day, mice received three flakes of oats containing 200mg/kg RU38486 or vehicle at 0900h. One hour after consumption at t=60 min, a blood sample was taken. Then, mice returned to their home cage or swam for 1 minute. Subsequent blood samples were taken at t=*90*, 120, *180* and 240 min after swimming. *Italic time points* indicate separate groups of mice. Between blood sampling, mice remained in their home cage.

Separate groups of mice were fed with Oats + RU38486 or Oats + VEH (n = 6/treatment) and blood samples were collected: (1) 15 min after oat delivery to estimate a possible short-lasting rise in corticosterone secretion due to oat consumption; and (2) to

further assess the duration of GR antagonism: one day before (control) and 8 hours after oat delivery during the circadian evening surge (at 1700h, i.e., two hours before lights off, (Dalm et al. 2005), and 24 hours after oat delivery.

Statistical analysis

Data was analyzed by one- or two-way analysis of variance (ANOVA; factors: treatment and/or condition), when appropriate with repeated measurements followed by Tukey *post-hoc* test. Total corticosterone values (AUC: area under the curve) were compared by t-test. Data are presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Experiment 1: Methods of drug-delivery and HPA axis activity

At baseline, corticosterone concentrations of all groups were in the range of low basal levels (Table 1: $F_{(5,55)}$=0.649, p = 0.663). Depending on the applied procedure, corticosterone concentrations increased after 30 and 60 min (time*group: $F_{(10,100)}$=11.406, p = 0.001). However, none of the procedures related to oat administration, nor eating of pure and vehicle-treated oats and procedures related to gavage and intraperitoneal delivery altered the plasma corticosterone concentration.

Corticosterone secretion in response to the vehicle delivered via oats, gavage and intraperitoneal injection increased over time and depended on the method of delivery (time*group: $F_{(4,18)}$=9.731, p = 0.001). While vehicle delivery via oats had

Table 1:

Basal morning resting and procedure-induced corticosterone concentrations (ng/ml) in blood plasma at 30 and 60 min after delivery. Data are presented as mean ± S.E.M.; $p < 0.05$: * **bold** vs. all other procedures and time points; *italic* vs. basal.

Delivery procedure	basal	30 min	60 min
pure oats	7.0 ± 0.8	8.8 ± 1.1	7.3 ± 1.1
oats + vehicle	8.3 ± 1.2	9.3 ± 1.2	7.5 ± 2.3
procedures – oats	6.3 ± 0.4	6.9 ± 0.6	5.4 ± 0.6
i.p. injection (vehicle)	7.9 ± 0.9	**115.8 ± 16.8***	**90.1 ± 16.3**
gavage injection (vehicle)	7.8 ± 1.2	**133.9 ± 36.8***	**43.3 ± 9.8**
procedures gavage and i.p.	8.1 ± 0.9	*25.0 ± 1.9*	*31.3 ± 14.2*

no effect, gavage and intraperitoneal injections resulted in significant elevations of corticosterone at 30 and 60 min (p = 0.001 and p = 0.005, respectively).

Blood glucose levels in response to oat consumption

Basal levels of blood glucose (8.53 ± 0.38mmol/l) were in the expected range for C57BL/6 mice at 10 weeks of age (Saravia et al. 2002). We observed that mice consumed the oats within 10 min after presentation. Blood glucose increased in response to oat consumption (t=5 and 10 min: 9.70 ± 0.30 and 11.46 ± 0.21mmol/l) and remained at the same level from 10 to 60 min after oat presentation (time: $F_{(3,35)}$=20.700, p = 0.001).

Experiment 2: Dose-controlled corticosterone administration via oats

Exogenous corticosterone delivered via oats dose-dependently increased plasma corticosterone concentrations (Figure 2: treatment ($F_{(1,21)}$=97.941, p = 0.001). The significant time*treatment interaction effect ($F_{(4,84)}$=20.865, p = 0.001) is due to the ADX group: ADX mice that received Oats + VEH had the expected low basal corticosterone concentrations of 5.90 $\pm$ 1.22ng/ml over all time points (p > 0.05). Consumption of oats containing corticosterone resulted in elevated concentrations at 30 and 90 min (p < 0.05): the dose of 15mg/kg corticosterone resulted in a 3-fold higher plasma concentrations than 4.5mg/kg (p < 0.05). When compared to 4.5mg/kg corticosterone and vehicle, corticosterone levels after 15mg/kg corticosterone were still significantly elevated 3 hours after consumption (p = 0.001).

Experiment 3: GR antagonist RU38486 delivery via oats

Mice consumed oats containing RU38486 or vehicle (treatment), one hour before swimming or not swimming (condition). Figure 3 depicts that corticosterone concentrations were significantly affected by treatment ($F_{(1,28)}$=701.424, p = 0.001) and condition ($F_{(1,28)}$=10.463, p = 0.001). In all but the VEH no-swim group, plasma corticosterone concentrations increased over time (time effect: $F_{(5,140)}$=65.825, p = 0.001). Corticosterone was significantly higher in the RU38486 than in the VEH mice (treatment*time $F_{(5,140)}$=57.058, p = 0.001) and significantly elevated by swimming (condition*time $F_{(5,140)}$=2.453, p = 0.036).

Swimming further potentiated the corticosterone concentrations (condition: $F_{(1,14)}$=4.667, p = 0.049), also expressed by significantly higher AUC values (mean ± SEM mg/ml; swim: 69.18 ± 3.29 vs. no-swim: 58.16 ± 2.94; p = 0.026). Corticosterone

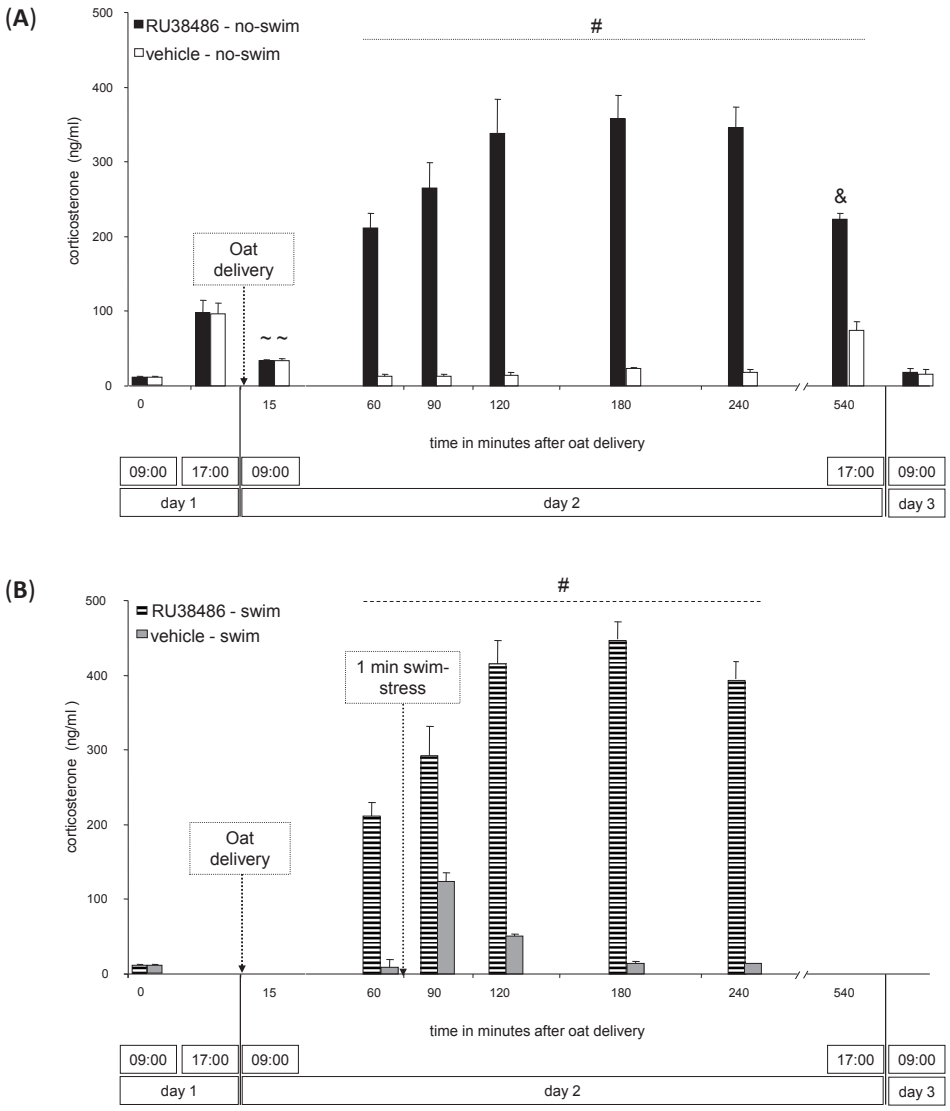

Figure 3

Plasma corticosterone in ng/ml before (day 1 at 0900 and 1700h) and after consumption of oat with the GR antagonist RU38486 or vehicle (indicated by arrow at 0900h) on days 2 and 3. Mice of both groups were **(A)** not exposed to a 1-min swim or **(B)** exposed to a 1-min swim stress (indicated by the arrow at 1000h), 60 min after consumption of the oats. Blood samples were taken from different groups of mice t=15, 60, 90, 120, 180, 240 and 540 minutes (1700h) after oat-consumption on day 2. On day 3, another sample was taken at 0900h. Data are presented as mean ± S.E.M.; $p < 0.05$: ˜ t=15 vs. basal: t=0 day 1 at 0900h; # RU38486 vs. VEH; & day 2 vs. day 1: evening corticosterone at 1700h.

concentrations before testing were low and comparable between the groups (data not shown; $F_{(3,31)}$=0.560, p = 0.646).

Already the consumption of RU38486 containing oats increased plasma corticosterone (Figure 3A; t=60 min after oat delivery; $p < 0.05$). The additional 15 min time point after oat consumption revealed a slight increase in corticosterone secretion in both RU38486 and VEH groups (vs. baseline: p = 0.001). Apparently preventing GR action at this time underlies the massive subsequent increase in plasma corticosterone in the RU38486 mice, while corticosterone returns to baseline values in the VEH group with intact GR function.

Swimming alone increased the total corticosterone values calculated as AUC (Figures 3A/B; vehicle groups: swim 7.41 ± 0.70 vs. no-swim 3.10 ± 0.27mg/ml; p = 0.001). Interestingly, 180 min after swimming, corticosterone levels were even lower than in no-swim controls (p = 0.013), and comparable to controls at 240 min.

RU38486 delivery via oats kept the levels of plasma corticosterone elevated for at least 8 hours as the evening concentrations (1700h) were still higher compared to VEH (p = 0.001) and the evening value one day before the oat delivery (p = 0.001). Comparably low resting corticosterone concentrations were found 24 hours after delivery of oats ($p > 0.05$).

Discussion

We have devised a non-invasive stress-free method of drug delivery in mice by validation on the glucocorticoid stress-system activity. Here we demonstrate that (1) ligands of the glucocorticoid system can be delivered via oats, (2) the effects are not confounded by long-lasting stress system activation induced by the method of administration and thus allow (3) drug delivery in close context with a test situation, e.g., a behavioral task. Furthermore, the procedure is easy to perform which minimizes variability of drug effects induced by the researcher and drug-application technique.

Any disturbance in the homeostasis of the organism induces HPA axis activity, which is expressed as an increase in circulating concentrations of corticosterone in mice and rats (De Kloet et al. 1998). The magnitude and duration of HPA axis activation is an accepted indicator for the degree of stress applied. In the present study, procedures related to drug delivery via oats did result in a minute increase in corticosterone. In contrast, procedures related to both, gavage and intraperitoneal injections resulted in substantial and long-lasting increased corticosterone concentrations. Whereas high-long

lasting increases of corticosterone are generally considered as “stressful”, we defined the minute increase in corticosterone as “stress free”. The devised method of drug delivery via oats reduced the magnitude and duration of stress system activation, could and should be used for other compounds as well.

Method of drug delivery

Non-invasive drug delivery in rodents can be realized via food or water. Mice are nocturnal animals, which mainly eat and drink during their behaviorally active (dark) period. Next to the fact that the dose of the drug delivered via free access to consumables cannot be controlled, most laboratories perform experiments during the inactive (light) period of the mice. To force dose- and time-controlled consumption, depriving mice of food and water is in itself a stressor (metabolic stress) with wide-spread consequences, also on HPA axis activity and disturbance of circadian activity patterns (Sommerville et al. 1988; Duclos et al. 2005). To avoid food deprivation-induced stress and to motivate eating during the light period, we selected oats as a treat. Mice like eating oats and readily overcome neophobia. Providing the oats at a fixed location in the home cage excluded stress induced otherwise by a novel environment or touch by a human experimenter. In the present study, all mice eat the oats containing the different glucocorticoid ligands within 10 min. The period of corticosterone and GR antagonist delivery via oats can be extended for at least one week (V. Brinks, S. Dalm, *unpublished*). However, drugs might have a bad taste or smell, such that mice might not eat the drug-containing oats (or only once). Hence, we recommend testing a possible neophobia or taste-aversion response to the oats + drug, like we have done, before the start of the experiment.

Choosing the appropriate vehicle reduces masking of wanted effects of administered drugs of interest. The selection of the most appropriate vehicle is based on the properties of the substance under investigation. Brown and colleagues (Brown et al. 2000) tested several vehicles, including water, corn oil and 1% methylcellulose/0.2% Tween80. They demonstrated that gavage administration of corn oil at 20ml/kg induced a stress response in a volume dependent fashion, whereas water and 1% methylcellulose/0.2% Tween80 did not. We also showed that the dissolvent of the glucocorticoid antagonist RU38486 in oats did not influence corticosterone concentrations.

To assure dose-controlled delivery via oats, mice are housed solitary. Housing conditions can significantly influence the behavior of mice, and this relates to enriched vs. poor environment, single vs. group housing, gender and strain effects (Ouagazzal et al. 2003; Chourbaji et al. 2005). Male mice, due to their territorial aggression should be

preferentially housed solitary with some environmental enrichment like paper towels (Van Loo et al. 2004). Social housing is the optimal way of housing female laboratory mice (Van Loo et al. 2007). To allow dose-controlled consumption of oats, introducing separations into the home cage of group-housed mice might be an option, but could induce stress due to disturbance of the home-environment as well. How feasible such a procedure is, has to be tested.

Effects of glucocorticoid ligands

Corticosterone. Administration of different doses of corticosterone to adrenalectomised mice allowed us to mimic the natural surge of corticosterone that has been described in response to a novel cage (Grootendorst et al. 2001b). The higher dose of 15mg/kg induced an approximate 3- fold increase in blood corticosterone concentrations when compared to the lower dose of 4.5mg/kg. Interestingly, 5 hours after administration of both doses, plasma corticosterone concentrations were similar. These findings will allow to choose appropriate doses of corticosterone in future studies. Based on the action mechanism of corticosterone (De Kloet et al. 1998) we may assume that in adrenally intact mice the two doses of corticosterone will have activated GR to a different degree, leading to enhanced negative feedback. Here, in ADX mice we may conclude that corticosterone also initiated GR actions. The decrease in plasma concentrations, however, is due to clearance from the organism, for both doses of corticosterone within 3 to 5 hours.

Glucocorticoid antagonist. In the current study RU38486 was administrated systemically one hour before a 1 minute swim trial. Given the corticosterone response, swimming strongly activates the HPA axis and can be considered as a stressor (Figure 3B). Mice that received RU38486 and swam for 1 minute, indeed showed the expected increase in corticosterone concentrations, lasting at least 8 hours, but less than 24 hours. Surprisingly, mice that consumed the RU38486-treated oats, but did not swim, also showed strongly increased corticosterone concentrations. This is in contrast to previous studies using intracerebral injections in rats showing that the effects of GR antagonism on corticosterone regulation occur in response to stress and at the peak of circadian corticosterone secretion, but not during the period of low basal corticosterone secretion (Ratka et al. 1989; van Haarst et al. 1996). Also systemic injections of GR antagonists in rats did not change basal corticosterone secretion (Spencer et al. 1998; Spiga et al. 2007). There are known species-dependent stress responses, and mice and rats habituate differently to laboratory handling and injections procedures (Balcombe et al. 2004). We assumed that oat-consumption itself might have activated the HPA axis within

this 30 min time frame and verified this in a follow-up experiment. Indeed, 15 min after oat consumption corticosterone concentrations were slightly increased. The slightly elevated blood glucose within 10 min is in accordance with the post-prandial increase in plasma glucose seen after consumption of carbohydrate-rich food. Glucocorticoids and glucose strongly interact (Gagliardino et al. 1984; Peters et al. 2004). We suggest that already the rather slightly elevated corticosterone will activate some GR to regulate negative feedback and thereby controlling the oat-consumption-induced secretion of corticosterone. The systemic application of the GR antagonist RU38486 involves an effective GR blockade throughout the entire organism and corticosterone levels kept rising. We conclude that the loss of GR activation potentiated corticosterone secretion due to the combined actions of glucose and corticosterone. This and the question of a genomic or non-genomic (via nuclear or membrane receptors) action of the GR antagonist cannot be resolved at present and should be addressed in further studies. The fast time course (within 1h) might point to a non-genomic action, however Morsink et al. (Morsink et al. 2007) reported in hippocampal tissue of rats genomic actions of corticosteroids via GR. Relevant is the fact that the initially unexpected increase of corticosterone due to oat consumption now additionally proves the efficacy of RU38486 as GR antagonist.

The dose of 200mg/kg RU38486 used was 16-fold higher compared to the study of Ratka (Ratka et al. 1989) and might be considered as extremely high. However, high doses of RU38486 prove very successful in patients suffering from neuropsychiatric disorders (up to 2000mg/BW (DeBattista and Belanoff 2006). Furthermore, mice lack the α_1-acid glycoprotein, which in humans binds about 95% of circulating RU38486 (Heikinheimo et al. 1987; Heikinheimo et al. 1989). Thus in mice, low concentrations of RU38486 are rapidly cleared from the body. In order to understand the mechanism underlying the beneficial effects of long term GR antagonism on endocrine and behavioral regulation, high dose of RU38486 will increase the likelihood of GR antagonism and as a consequence disturb negative feedback. This is clearly demonstrated by the long-lasting elevation of endogenous corticosterone. While exogenous corticosterone is cleared from the system after 3 to 5 hours, corticosterone levels remain extremely high during this time domain. This is indicative for continuous secretion of corticosterone due to the blockade of negative feedback actions via GR (Dalm 2006).

With respect to the use of oats as a reward or carrier for drugs in studies on learning and memory, we have to keep in mind that glucose is also known to modulate cognitive functions (Messier 2004). However, to be effective, glucose has to be administered in much higher concentrations than induced by oat consumption.

Moreover, the number of oats could be reduced to minimize effects on corticosterone and glucose secretion.

Drug delivery via oats has several advantages over other methods of application. It is (i) non-invasive; the short-lasting slight increase in plasma corticosterone after oat consumption, is not comparable to injection-induced effects, neither in quantity nor quality; (ii) easy to use; (iii) allows administration in close-context and can be used repeatedly; (iv) no food or water deprivation is needed (Mitev et al. 1993); (v) time of administration is not confined to the dark, behaviorally active period; (vi) the vehicle is inherent to the drug effects; (vii) it resembles more the conditions in human drug delivery. Of course, there are also arguments against this kind of peripheral drug delivery. First, the drug effect is not selective as it reaches the entire body and may not pass the blood-brain-barrier. This holds true for other systemic methods of drug delivery and is also related to pharmacodynamic and –kinetic characteristics of the drug. Second, timing of drug-delivery will depend on the number of oats presented, but can be achieved within 10 min or less. It might not be suitable to study fast drug effects, while it does allow to study drug effects in close-context to, for instance, behavioral performance. Third, administration via oats requires that mice are single housed, at least during the time of drug delivery. Separating mice with partitions in their home cage might be a possibility. However it is likely, that interference with the home cage environment will introduce an extra stress factor. It might be possible to adapt mice to such handling by a series of habituation trials. Fourth, taste and smell of the drug could influence its consumption, but these are aspects that have to be tested before the experiment. When to use this method of drug delivery via oats? One has to balance the pros and cons, but it is the scientific question that is central to the design of the experiment.

Conclusions

We consider drug delivery via oats as method of choice as it allows to dissociate the effect of the administration procedure from the properties of a drug. Since we have demonstrated that intraperitoneal and gavage injections lead to long-lasting corticosterone exposure that most likely will affect memory processes (Sandi et al. 1995; Sandi et al. 1997), we specifically propose oats for context-dependent stress-free drug delivery.

Acknowledgements

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Chapter 4

Paradoxical decrease in basal and stress-induced corticosterone secretion after recurrent daily blockade of the glucocorticoid receptor

Sergiu Dalm
E. Ronald de Kloet
Melly S. Oitzl

Being prepared for submission

Abstract

The present study tests the hypothesis that repeated daily administration of the glucocorticoid receptor antagonist mifepristone (MIF) would lead to chronic disinhibition of the hypothalamic-pituitary-adrenal axis. Male C57BL/6J mice were offered 200 mg/kg MIF *per os* in oats, either once (1xMIF) or daily for seven days (7xMIF), or vehicle. Plasma corticosterone levels were determined in blood samples obtained i) at various time points after MIF intake; ii),immediately following five min exploration of a circular hole board at 24h after the first and seventh administration. At that time the mice were sacrificed for quantification of mineralocorticoid (MR) and glucocorticoid receptor (GR) mRNA expression in the hippocampus and the paraventricular nucleus (PVN).

After 1xMIF corticosterone levels were elevated for about 16h, and then decreased towards vehicle control levels at 24h, while showing a much higher corticosterone response to circular hole board exposure. Following 7xMIF the basal and stress-induced corticosterone patterns were comparable to vehicle. The 1xMIF mice showed behavioral hyperactivity during exploration of the circular hole board, while the 7xMIF mice rather engaged in serial search patterns. MR mRNA was decreased in all hippocampal subregions of the 1xMIF group, and increased in the 7xMIF group only in the CA2 cell field. GR mRNA expression in hippocampus and PVN was not affected. Adrenal weights were increased in both MIF groups.

In conclusion, the data show that after recurrent blockade of GR the adrenal corticosterone secretion is downregulated rather than disinhibited because of intermittent glucocorticoid feedback, while MR-dependent characteristics become prominent in exploratory behavior.

Introduction

It has been reported that patients suffering from psychotic major depression benefit from a brief treatment with the glucocorticoid antagonist RU38486, also known as mifepristone (MIF; 600 – 1200mg/day, once a day for four to seven days). These high doses of the antiglucocorticoid improved emotional and cognitive abilities and restored aberrant levels of corticosteroids (Murphy et al. 1993; Belanoff et al. 2001a; DeBattista and Belanoff 2006; Flores et al. 2006; Blasey et al. 2009; Blasey et al. 2011). The fast amelioration of psychotic and depressive symptoms is thought to be at least in part due to restoration of glucocorticoid action to which untreated depressed patients are resistant, while the anti-progestin activity of MIF seems not implicated (Belanoff et al. 2001a; Thomson and Craighead 2008).

In the present study we asked how daily administration of MIF would affect the secretion of corticosterone. Recent studies have addressed this question but have provided different results. Wulsin et al. (Wulsin et al. 2010) using a daily dose of 10mg/kg of MIF in rats found attenuated basal and stress-induced Hypothalamic-Pituitary-Adrenal (HPA) axis activity and attenuated depression-like behavior. Bachman et al. (Bachmann et al. 2003) offered rats 20mg/kg/day of various GR antagonists added to food and observed episodic increases in HPA axis activity and a profound increase in hippocampal MR expression (see also Spencer et al. 1998; Oomen et al. 2007) gave twice a day MIF orally (50mg/kg rat) to chronically stressed rats and found that this treatment blocked the stress-induced reduction in neurogenesis. Revsin et al. (Revsin et al. 2009) also administered twice a day MIF orally (200mg/kg mouse) and observed elevated HPA axis activity. Finally, van Haarst et al. (van Haarst et al. 1996) infused MIF chronically 100ng/hr intracerebroventricularly in rats and observed after 3 days infusion enhanced stress-induced and circadian rises in corticosterone secretion under conditions in which spatial memory was improved (Oitzl et al. 1998). Common to these studies was that adrenal weight and responsiveness to ACTH were increased indicating that the changes were due to MIF's anti-glucocorticoid rather than anti-progestin activity. Based on these results we tested therefore the hypothesis that daily administration of a very high dose of MIF would disinhibit the HPA axis.

In the current study we mimicked in naive male C57BL/6J mice the high dose regimen of MIF common for the patient studies. For this purpose we applied a previously devised non-invasive stress-free method for steroid delivery via oats (Dalm et al. 2008). After the first (1xMIF) and the seventh administration (7xMIF) of the glucocorticoid antagonist or vehicle we assessed (i) in 2h-intervals the circadian corticosterone secretion

pattern; (ii) 24h post-treatment the behavioral and corticosterone response to novelty during five min exploration of a circular hole board. Immediately thereafter mice were sacrificed and hippocampal and hypothalamic MR, GR and CRH mRNA expressions were measured in brain sections with *in situ* hybridization. The data demonstrated contrary to our hypothesis that recurrent GR blockade with MIF downregulates HPA axis activity, while altering the behavioral response to novelty.

Materials and Methods

Animals

Male C57BL/6J mice, 8-10 weeks of age, were purchased from Janvier (France). Upon arrival at the animal facilities (Gorlaeus Laboratory, LACDR, University of Leiden, The Netherlands), mice were single housed in a temperature (21 ± 1˚C) and humidity (55 ± 5%) controlled room, with food and water *ad libitum;* for ten days before the start of the experiment (12-12h light-dark cycle; lights on 0700 to 1900h). During this period mice were weighed and handled every other day. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Study design

The experiments were conducted with separate groups of mice. We measured: (1) the 24h circadian corticosterone secretion, following single and repeated administration of MIF (200mg/kg; 1x/day for seven days). In addition, we collected blood samples around the time of the circadian corticosterone peak 32h after the last administration of MIF; (2) corticosterone concentrations before (basal) and after five min of novelty exposure to the circular hole board during which behavior was recorded for further analysis; (3) following behavioral testing, mice were decapitated and brains were prepared for measuring the expression levels of MR, GR and CRH mRNA in the hippocampus and paraventricular nucleus of the hypothalamus (PVN).

Procedures

Familiarization of mice to oat administration and drug delivery procedures as described in (Dalm et al. 2008) are applicable to all experiments of this study.

Familiarization to oat administration

One week prior to the start of the experiment a feeding-cup (2.3cm diameter x 2.5cm high) was taped to the floor in a corner of the home cage, opposite the nest location. For familiarization, three flakes of oats (Speltvlokken, Biologische teelt, Graanpletterij de Halm, Netherlands; ± 140mg) were placed in the cup on days 1, 3 and 5 of week 1, 2 hours after lights on. The top of the home cage was lifted and the sawdust was removed from the cup using an air puff generated with a pipette. Next, the oats were placed into the cup using forceps to minimize human odor transfer. Thereafter, the home cage was closed and the mouse was allowed to eat the oats undisturbed. All the oats were consumed within 10 min.

Drug delivery

Preparation of drug delivery via oats: One day prior to the experiment three flakes of oats were placed in a glass vial and the solutions containing GR antagonist or dissolvent (VEH) were applied. The glass vials containing the oats were kept at room temperature over night. Within 16h, the solution was absorbed by the oats and they were dry when presented to the mice.

The GR antagonist mifepristone (MIF) (kindly provided by Corcept Pharmaceuticals, CA, U.S.A.) was dissolved in 1 ml 0.9% NaCl containing 0.25% carboxymethylcellulose and 0.2% Tween20 (VEH=dissolvent). From this solution 50µl was applied to the oats (mice received a dose of 200mg/kg MIF).

Hormone assays

The circadian corticosterone concentrations were measured in blood samples obtained via tail incision (Dalm et al. 2005). Briefly, a small incision with a razor blade at the base of the tail allowed collection of 50µl blood within 90s after opening of the animal's cage. Following decapitation, trunk blood was collected individually in capillaries coated with potassium-EDTA (Sarstedt, Germany), stored on ice, and centrifuged with 13000 rpm at 4°C for 10 min. Plasma was stored at –20°C. Corticosterone concentrations were measured using commercially available radio immunoassay kits ^{125}I-corticosterone (MP Biomedicals, Inc., NY, USA; sensitivity 3 ng/ml).

Experiment 1: Effect of GR antagonism on corticosterone secretion

Animals

Mice (n = 54) were randomly assigned to three treatment groups (n = 18/group): (1) single mifepristone (1xMIF); (2) MIF once a day on seven consecutive days (7xMIF) or (3) VEH on seven consecutive days (VEH). Oats+MIF or Oats+VEH were placed in the feeding cup at 0900h, and consumed within 10 min.

Experimental design

The circadian corticosterone secretion was determined in blood samples collected via tail incision every two hour over a period of 24h. The first blood sample was taken at 1100h, i.e., two hours after MIF or VEH was administrated, and the last at 0900h the next day. Subsequent blood samples were collected starting 32h after the last administration around the circadian corticosterone peak at 1700, 1900, 2100 and 2300h.

The three treatment groups were divided in three subgroups each, consisting of six mice. Thus, from each mouse, one blood sample was taken every six hours and each time point consisted of six mice per group. During the dark period, blood sampling took place under red light conditions.

Experiment 2: Corticosterone and behavioral responses to the circular hole board

Animals

Mice (n = 24) were randomly assigned to three treatment groups (n = 8/group): (1) single mifepristone (1xMIF); (2) mifepristone once a day on seven consecutive days (7xMIF) or (3) VEH on seven consecutive days (VEH). Oats+MIF or Oats+VEH were placed in the feeding cup at 0900h, and consumed within 10 min.

Experimental design

Twenty-four-hours after the last administration of MIF or VEH we took a blood sample via tail incision, and placed the mouse for 5 min on the circular hole board; the behavioral response was analyzed. Immediately following behavioral testing, mice were decapitated. Corticosterone concentrations were determined in trunk blood. Brains were snap frozen in isopentane, pre-cooled on dry ice/ethanol and stored at –80°C until further use, i.e. to determine MR, GR and CRH mRNA expression levels in brain tissue. Thymus and adrenals were removed and weighed.

Circular hole board

Apparatus: A grey round plate (Plexiglass; 110cm diameter) with 12 holes (5cm diameter, 5cm deep) at equal distances from each other, and at a distance of 10cm from the rim of the hole to the rim of the plate, was situated one meter above the floor in a different experimental room then the housing room. Light conditions on the surface of the board were 120lux. To minimize, and distribute odour cues, the surface was cleaned with 1%HAc and the board was turned (randomly clock- and anticlockwise) before a mouse was tested. Behavior was recorded on videotape and analyzed with an automated tracking system (Ethovision 3.1, Noldus Information Technology, Wageningen, The Netherlands). The position of the mouse was sampled five times per second. To calculate the distance walked, we set the minimal distance between samples to 3cm. The following parameters related to general activity, exploratory strategies and possible anxiety-related behaviors were analyzed: distance walked (m) on the board (=total arena) and in specified zones defined as: start center = circle of 30cm diameter, rim zone = a ring of 4.5cm at the outer perimeter of the plate. Parameters: velocity (cm/s), number of holes visited; sequence of hole visits (*serial*: more than two holes in sequence; *perseveration*: repeatedly visiting the same hole or alternately visiting two neighbouring holes); latency (s) to leave the center; latency (s) to and time spent (s) in rim zone.

In situ hybridization for MR, GR and CRH mRNA

Brains were sectioned at –20°C in a cryostat microtome at 10µm in the coronal plane through the level of the hypothalamic paraventricular nucleus (PVN) and dorsal hippocampus. Sections were thaw-mounted on poly-L-lysine coated slides (0.001%), air dried and kept at –80°C until further use.

In situ hybridizations using ^{35}S-labeled ribonucleotide probes (MR, GR, CRH) were performed as described previously (Schmidt et al. 2003). Briefly, sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense RNA probes were transcribed from linearised plasmids containing exon-2 of mouse MR and GR, and the full length coding regions of CRH (rat). Tissue sections (3–4/slide) were saturated with 100µl hybridization buffer containing 20mM Tris-HCl (pH 7.4), 50% formamide, 300mM NaCl, 1mM EDTA (pH 8.0), 1x Denhardt's, 250 µg/ml yeast transfer RNA, 250 µl/ml total RNA, 10mg/ml salmon sperm DNA, 10% dextran sulfate, 100mM dithiothreitol, 0.1% SDS, 0.1% sodium thiosulfate and supplemented with approximately 1.5 x 10^6cpm ^{35}S-labeled riboprobe. Brain sections

were cover slipped and incubated overnight at 55°C. The next day sections were rinsed in 2xSSC, treated with RNaseA (20mg/ml) and washed in increasingly stringent SSC solutions at room temperature. Finally, sections were washed in 0.1xSSC at 65°C for 30 min and dehydrated through increasing concentrations of ethanol. All age groups were assayed together. Films were opposed to Kodak Biomax MR film (Eastman Kodak Co., Rochester, NY) and developed.

Autoradiographs were digitized, and optical density of the areas of interest was quantified using image analysis computer software (analySIS 3.1, Soft Imaging System GmbH). The average density of six measurements for each animal was calculated.

Statistical analysis

The circadian profile of corticosterone was analyzed by analysis of variance (ANOVA - factor: treatment) with repeated measurements, followed by LSD *post-hoc* test. Total corticosterone (AUC: area under the curve) over 24h was calculated for light and dark periods of 12h, subjected to ANOVA, with treatment and time of the day as fixed factors. Body-, adrenal-, and thymus weights were analysed using one-way ANOVA followed by Bonferroni's multiple comparison *post hoc* test. Data are presented as mean ± S.E.M. Statistical significance was accepted at $p < 0.05$.

Results

Experiment 1: Effect of GR antagonism on corticosterone secretion

Circadian pattern of plasma corticosterone level

Mice of all groups showed a circadian corticosterone rhythm (Figure 1A; time $F_{(11,165)}=35.051$, $p < 0.001$) as previously described (Dalm et al. 2005). The corticosterone secretion of control mice increased from 1500h onwards, with peak levels (± 100ng/ml) at the end of the light phase and the beginning of the dark phase (between 1700 and 2100h). Interestingly, the frequency of MIF administration affected the course of the circadian rhythm (time*group: $F_{(22,165)}=15.992$, $p < 0.001$). Corticosterone concentrations in 1xMIF-mice were significantly higher from 1100 until 0100h ($p < 0.01$), reaching and maintaining peak levels from 1300 until 2300h (± 300ng/ml). Around 2300h, concentrations readily declined until there was no difference in corticosterone concentration at 0300h vs. control and 7xMIF-administrated mice. There was a sudden significant increase vs. controls ($p = 0.001$) and 7xMIF mice ($p = 0.013$), at 0500h. In contrast, repeated MIF

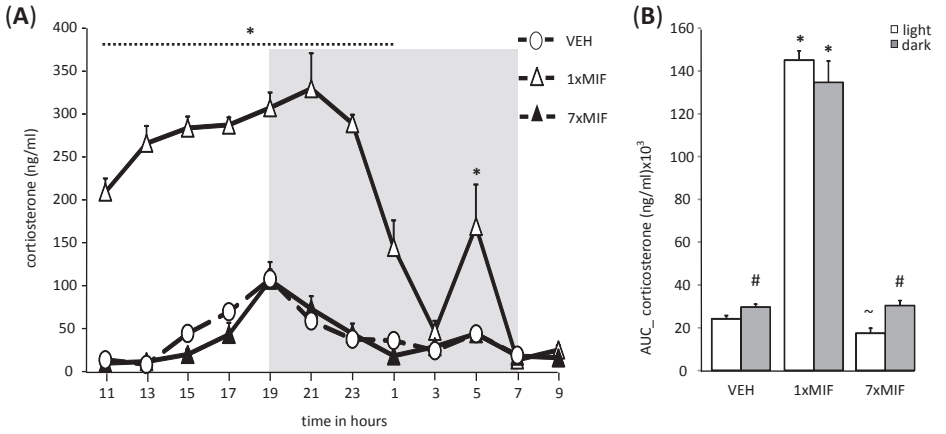

Figure 1

(A) Circadian secretion of corticosterone in ng/ml measured every 2 hours in blood plasma of male mice C57BL/6J that received RU38486 (MIF) once (1xMIF) or for seven days (7xMIF). Mice were entrained in a 12-12h light-dark cycle (dark phase from 1900 to 0700h represented by the gray shaded area). **(B)** Total corticosterone secretion in ng/ml during the light and dark period of the day, determined as Area Under the Curve (AUC); ng/ml. Data are presented as mean ± S.E.M; $p < 0.05$ * vs. other groups, # within groups, ˜ 7xMIF vs. VEH.

administration did not boost the concentrations of corticosterone as was observed for 1xMIF administrated mice; the time course was similar to VEH mice. Overall, there was a main effect of treatment due to the high corticosterone concentrations in the 1xMIF mice ($F_{(2,15)}$=550.923, $p < 0.001$).

Total amount corticosterone

The total amount of corticosterone calculated as area under the curve (AUC) over 24h showed a main effect of treatment (Figure 1B AUC: $F_{(2,17)}$=392.094, $p < 0.001$). AUC corticosterone during the dark period (1900 to 0700h) was higher than during the light period (0700 to 1900h) in VEH and 7xMIF mice (paired t-test; both $p < 0.01$). 1xMIF mice had similar high AUC corticosterone levels during the light and dark periods, both significantly higher than VEH and 7xMIF mice. Interestingly, AUC corticosterone was lowest during the light period of 7xMIF mice ($p < 0.039$ vs. VEH) due to the low corticosterone concentrations measured from 1500 till 1700h.

Corticosterone around the circadian peak, 32h after mifepristone administration

Treatment effects were found around the time of the circadian peak (Figure 2, 1700 to 2300h; $F_{(2,15)}$=6.308, p =0.01). Thirty-two hours after the last administration, 1xMIF mice secreted less corticosterone than VEH (p = 0.007) and 7xMIF mice (p = 0.008). No statistical difference was found for corticosterone secretion patterns of VEH and 7xMIF groups.

Experiment 2: Corticosterone and behavioral responses to the circular hole board

Basal and novelty induced corticosterone secretion

Basal resting as well as novelty induced corticosterone were affected 24h after the last treatment (Figure 3; treatment $F_{(2,44)}$=17.175, p < 0.0001; time $F_{(1,44)}$=45.980, p < 0.0001; treatment*time $F_{(2,44)}$=17.626, p < 0.0001). Basal resting corticosterone differed

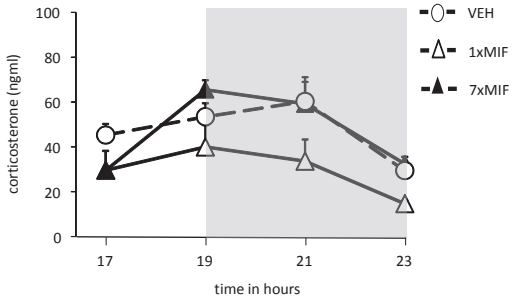

Figure 2
Corticosterone (ng/ml) secretion during the circadian peak in mice, 32h after last administration of RU38486 (MIF), 1xMIF, 7xMIF or VEH (dark phase from 1900 to 2300h represented by the gray shaded area). Data are presented as mean ± S.E.M.

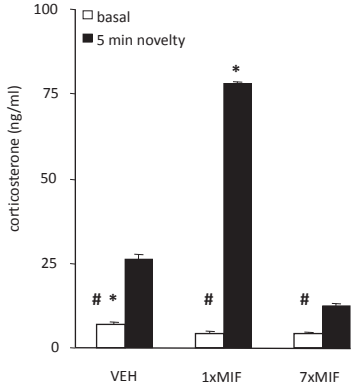

Figure 3
Basal and novelty (5 min exposure to the circular hole board) induced corticosterone (ng/ml) were determined in mice, 24h after last administration of VEH, 1xMIF or 7xMIF. Data are presented as mean ± S.E.M.; p < 0.05 * vs. other groups, # within groups.

significantly between the groups ($F_{(2,23)}$=14.656, p < 0.001) and was lower in both MIF treated groups than in VEH mice (p < 0.001). Basal corticosterone of 1xMIF and 7xMIF mice was comparable. After 5 min on the circular hole board, corticosterone was increased in all groups compared to baseline, however to a different degree ($F_{(2,23)}$=19.074, p < 0.0001). Corticosterone levels in 1xMIF where 300% of the VEH group and 700% of the 7xMIF group (both p < 0.0001); corticosterone of the VEH group was about twice as much as in the 7xMIF group (p < 0.05).

Expression of MR, GR, CRH mRNA in hippocampus and PVN

Hippocampal MR mRNA expression was differentially affected by treatment, 24h post-administration, across all subfields (Figure 4; treatment – DG: $F_{(2,23),}$=11.005, p = 0.001; CA1: $F_{(2,23)}$=12.887, p = 0.001; CA2: $F_{(2,23)}$=14.267, p = 0.001; CA3: $F_{(2,23)}$=11.550, p = 0.001). MR mRNA expression was reduced across all subfields in 1xMIF-mice compared to VEH and 7xMIF-mice (p < 0.05). Repeated MIF administration increased MR mRNA expression in the CA2 specifically vs. VEH and 1xMIF-mice (p = 0.016 and p = 0.001, respectively).

Neither GR nor CRH mRNA expression in hippocampus and PVN were affected by treatment (data not shown).

Exploration on the circular hole board

Twenty-four hours after administration the behavioral response differed during five min exploration on the circular hole board (Table 1: MANOVA: $F_{(20,26)}$=3.772, p = 0.001). Following initial slower movement out of the central start position, 1xMIF mice showed hyperactivity: they walked longer distances, with a faster speed of moving, visited more

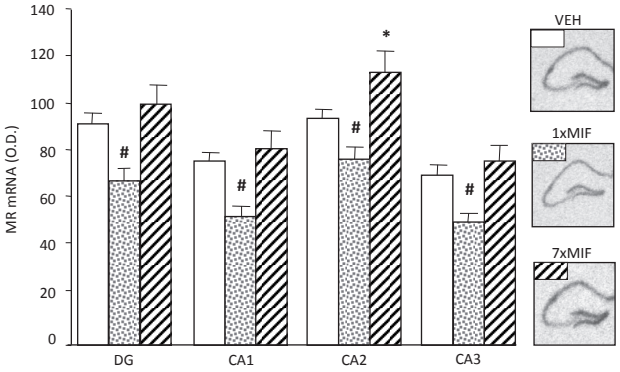

Figure 4
Expression of MR mRNA, measured as optical density (O.D.) in the hippocampal subfields dentate gyrus (DG), CA1, CA2 and CA3, 24h after last administration of VEH, 1xMIF or 7xMIF. Data are presented as mean ± S.E.M.; p < 0.05 * vs. other groups, # within groups.

Table 1: The behavioral response during five min circular hole board exposure, 24h after the last administration with RU38486 (MIF).

	VEH	1xMIF	7xMIF
General activity			
distance walked (m)	7.9 ± 0.7	***15.0 ± 1.9****	7.5 ± 0.9
speed of moving (cm/s)	8.6 ± 0.4	***11.6 ± 0.8****	9.9 ± 0.3
total hole visits	14.8 ± 2.1	***24.8 ± 1.8****	17.0 ± 2.4
Search strategy			
latency (s) from center	8.4 ± 1.4	***14.0 ± 1.4**$^{\#}$*	11.8 ± 2.1
latency (s) first hole visit	13.9 ± 0.8	16.0 ± 2.6	***18.4 ± 0.7**$^{\#}$*
%serial	16.5 ± 5.2	28.2 ± 3.6	**36.6 ± 10.2**$^{\#}$
%perseveration	48.6 ± 5.8	39.3 ± 5.2	52.1 ± 5.1
Anxiety-related			
latency (s) to rim	63.0 ± 13.1	55.1 ± 12.4	69.9 ± 8.5
number of rim dips	12.8 ± 1.4	***18.4 ± 2.0****	11.1 ± 1.2
number of boli	1.1 ± 0.7	0.8 ± 0.4	1.3 ± 0.8

Data are presented as mean ± S.E.M.; $p < .05$ * vs. other groups; # vs. VEH.
Bold italic indicates significant differences.

holes and made more rim dips (vs. VEH and 7xMIF-mice: $p < 0.05$). Interestingly, 7xMIF-mice made more use of a serial search strategy (vs. VEH-mice: $p = 0.05$).

Other physiological measures

Treatment did not influence body weight. Adrenal weight ($F_{(3,34)}$=3.733, $p = 0.035$) was highest in both MIF-groups, but significantly higher in 7xMIF than in VEH ($p = 0.005$): adrenals in mg, mean ± SEM: VEH 23.5 ± 2.8; 1xMIF 31.3 ± 4.3; 7xMIF 39.3 ± 2.9. Thymus weight was lower in both MIF groups, but passed statistical significance ($F_{(3,34)}$=3.100, $p = 0.059$): thymus in mg, mean ± SEM: VEH 411.0 ± 38.9; 1xMIF 371.5 ± 15.1; 7xMIF 321.1 ± 23.6.

Discussion

The present study demonstrated that single delivery of a very high dose of MIF caused a profound increase in circulating corticosterone levels starting 2h first after ingestion and lasting 16h before reaching vehicle control levels at 24h. At that time exposure to a novelty stressor still triggered a profound corticosterone response. This disinhibitory

effect exerted by the antagonist was in line with our expectation. We did not expect, however, that after one week of daily consumption of the same high dose of MIF the basal and stress-induced corticosterone levels were back to baseline concentrations not different from vehicle controls. Accordingly, the current data reject the hypothesis that daily repeated MIF administration would produce a state of hypercorticism. At the same time the weight of the adrenals was further increased, while the thymus weight progressively decreased towards significance. The increased adrenal weight suggests that in spite of the progressive downregulation of the HPA axis apparently still sufficient ACTH is released over the week to exert its corticotrophic action.

How does the slow downregulatory adaptation of adrenal corticosterone secretion come about? One obvious explanation is related to the recurrent nature of daily GR blockade with the antagonist. MIF is rapidly cleared because the antagonist is not bound to albumin, a high capacity low affinity binder and not α1-acid glycoprotein, which is a low capacity high affinity binder, and rapidly metabolized (Heikinheimo and Kekkonen 1993). We found in another study that after a 50mg/kg rat dose orally, MIF is already depleted from the circulation in 90 min, while low amounts of the antagonist and its metabolites are retained in the brain for at least 3 hours (Karssen, Belanoff and de Kloet, unpublished observation). Corticosterone remained elevated though for 16 hours, while the genomic effects will persist even longer. Our experimental design thus allowed us to study a recurrent pattern of GR-mediated actions including negative feedback which are transiently interrupted by daily application of the GR antagonist. We propose therefore that the HPA axis progressively adapts to this daily cycle of GR blockade and subsequent GR activation. Hence, during the seventh day of GR antagonist administration, the circadian and stress-induced corticosterone pattern had become similar to that observed in control mice.

This slow adaptation of the HPA axis to MIF has been observed before. The elegant study by Wulsin et al (Wulsin et al. 2010) revealed that a twenty fold lower dose of MIF *i.p.* (10mg/kg rat) produced an attenuated HPA axis response to a forced swim stressor, after one week. Interestingly, this course of MIF treatment also evoked a differential pattern of activation and inhibition of central inputs to the PVN. The ventral subiculum of the hippocampus and all regions of the medial frontal cortex showed enhanced stress-induced c-Fos activity after daily GR blockade, while the c-Fos response was reduced, however, in other subregions of the hippocampus and in the amygdala. These data suggest that MIF enhanced inhibitory and suppressed excitatory inputs to the PVN that collectively may contribute to downregulation of HPA axis activity.

Also the mode of MIF application is important. A previous study showed that if MIF was chronically infused in the cerebral ventricles using an Alzet minipump a constant blockade of all brain GR sites was achieved. After four days of infusion the amplitude in the circadian and stress-induced corticosterone patterns were gradually enhanced because the peak levels of corticosterone had become higher, while the troughs remained low and did not alter (van Haarst et al. 1996). In the present study using recurrent daily rather than chronic blockade of GR the opposite adaptation occurred: initially a large surge in circadian and stress-induced corticosterone secretion was observed which then subsided over the next days of GR antagonist oral administration. This finding is reminiscent to the behavioral dichotomy between repeated and chronic infusions of MIF. It was found that continuous blockade of brain GR facilitated spatial learning and memory of rats, while phasic blockade caused a deficit (Oitzl et al. 1998). It is conceivable that besides blocking feedback suppression in pituitary and PVN, the effect of MIF on limbic circuitry noted by Wulsin et al (Wulsin et al. 2010) underlies this *opposite* change in stress sensitivity and behavioral performance after phasic vs. continuous blockade of the GR.

The above noted dichotomy in behavior may develop because after phasic (one time per day) GR blockade by MIF the rebound corticosterone response exerts its effect including the feedback suppression of the HPA axis, while during continuous blockade such agonist corticosterone actions are excluded. It also seems that if the phasic blockade is stepped up to two times per day with a high dose of MIF the condition of continuous blockade is approached (Oomen et al. 2007; Revsin et al. 2009). However, irrespective of phasic or continuous GR blockade, corticosterone binding to the MR will always occur (Reul and de Kloet 1985; Wodarz et al. 1992; Calfa et al. 2003). MR is known to mediate control over appraisal processes, behavioral reactivity to novel experiences and the onset of HPA axis activity (De Kloet et al. 1998). The changes in corticosterone action via MR activation were reflected by the lower expression of hippocampal MR mRNA for the 1xMIF-group, whereas expression was increased in the CA2 region of the hippocampus for 7xMIF-group. Interestingly, GR blockade after MIF treatment *per os* for three weeks, increased total hippocampal MR mRNA expression by 1.5 compared to controls (Bachmann et al. 2003). Therefore, it would be of interest to determine longitudinal effects of repeated GR blockade on MR function, particularly since previous studies have clearly shown that MR and GR interact in the control of HPA axis activity (Herman and Spencer 1998; Spencer et al. 1998).

In the studies by Bachmann et al (Bachmann et al. 2003) and Wulsin et al. (Wulsin et al. 2010) chronic MIF affected forced swimming behavior in a similar manner to antidepressants. In another study by Oitzl et al (Oitzl et al. 1998) chronic MIF leaving

MR active improved memory performance. Studies with mouse mutants overexpressing MR in limbic forebrain revealed enhancement of memory (Ferguson and Sapolsky 2007; Lai et al. 2007), perseveration of learned behavior (Harris et al. 2012 under review) and reduction of anxiety (Korte et al. 1996). Previously, also the pharmacological blockade of MR had shown altered appraisal processes and selection of the appropriate behavioral response, i.e., search strategy (Oitzl and de Kloet 1992; Oitzl et al. 1994; Oitzl et al. 1997a). In the current study twenty-four-hours following repeated GR antagonism, mice used the serial search strategy more often, compared to controls. This strategy increases the likelihood that the animals will visit all possible escape routes that the circular hole board provides (during the exploration trial all holes were closed). Indeed, the choice of applied strategy does affect performance in spatial learning trials (Dalm et al. 2000; Grootendorst et al. 2001a). In the current study following 1xMIF mice were initially slower to move away from the start center, and subsequently hyperactive on the circular hole board. This could indicate a change in the level of anxiety induced by previous GR antagonism. If so, then the effect is transient as repeated GR antagonism did not induce any of the above described features.

In conclusion, based on the current findings the efficacy of GR antagonism in clinical studies could be due to the following factors: (1) The detrimental effects of high corticosteroid levels via GR activation are prevented by GR antagonism. This possibility is prominent during continuous or high frequency blockade of the GR. (2) If during daily cycles of MIF application, the blockade by the GR antagonist wanes, GR becomes activated by the high circulating corticosterone levels and shuts off its own secretion (Wodarz et al. 1992; De Kloet et al. 1998; Calfa et al. 2003). (3) As a result of GR blockade and the subsequent rise in corticosterone levels, the MR becomes strongly activated irrespective of phasic or continuous GR antagonism.

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Conflict of interest

MSO and SD report no conflict of interest. ERdK is on the Scientific Advisory Board & owns stock of Corcept Therapeutics.

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Chapter 5

Post-training self administration of sugar facilitates cognitive performance of male C57BL/6J mice in two spatial learning tasks

Sergiu Dalm
Lars Schwabe
Hartmut Schachinger
Melly S. Oitzl

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Abstract

Spatial memory can be strengthened by adverse stimuli that activate the stress system, and administration of the stress hormone corticosterone in close-context with the learning task. Less is known about modulation of spatial memory by post-training positive reinforcers (reward). Cognitive performance was assessed in male C57BL/6J mice using two learning tasks: the water maze (WM) and circular hole board (CHB). Sugar was chosen as a post-training reinforcer. We expected that the free access to sugar immediately (0h) after training would facilitate spatial memory; delayed access to sugar (4h after training) or no sugar served as controls.

In both tasks, 0h-sugar mice showed superior performance, indicated by shorter latencies and distances to the trained spatial location. The memory facilitating effect of sugar became visible at distinct times during training: on the CHB from the first trial onwards, in the WM on training days 4 and 5. Sugar-rewarded mice kept their superior performance during the free exploration/swim trial, expressed by more persistent search strategies for the exit hole or platform. Post-training sugar reward in close-context with performance strengthens memory via modulation of consolidation.

These findings supports the integrative theory of reinforcement and memory. We suggest that our experimental set-up will allow to differentiate between direct effects on memory and alterations in reward processes in animal models of stress-related diseases.

Introduction

Memory formation is modulated by task inherent appetitive and aversive characteristics. Other stimuli occurring in close context with the task either impair or enhance memory (Dawson and McGaugh 1971; McGaugh and Herz 1972). Decades ago, Huston and colleagues presented a memory processing theory of reinforcement, proposing that the reinforcer acts on a memory of the response or of the stimulus-response contiguity (Huston et al. 1974; Huston and Mondadori 1977). It has provided a framework for studies that have demonstrated a close correspondence between memory promoting and reinforcing effects of natural reinforcers like food, but also of electrical and chemical stimulation of the brain (Huston and Oitzl 1989).

Here we address the effect of a post-training natural reinforcer on cognitive performance in two spatial learning paradigms in mice: the well known and commonly used water maze (WM; Morris 1984), and the circular hole board (CHB; Barnes 1979). Both tasks have been originally designed for rats. Mice prefer dry-land over wet mazes (Whishaw 1995; Whishaw and Tomie 1996; Wotjak 2004). For mice, the degree of the task-inherent aversive characteristics differs largely (Wotjak 2004), in parallel with the activation of the stress system and secretion of glucocorticoid hormones (De Kloet et al. 1998; Joels et al. 2006). For example, increasing the aversiveness of the task, like lowering the water temperature in the water maze, increases the secretion of the stress-hormone corticosterone and results in memory improvement in rats (Sandi et al. 1997; Akirav et al. 2001). Injections of corticosterone have comparable effects on memory (for review Joels et al. 2006). The WM is regarded as life-threatening while the CHB is considered to be less (or not) aversive, as the animal walks to locate a hole leading to its home cage. Thus, modulation of the adverse components of a task facilitates learning and memory processes (e.g., lowering water temperature, increasing strength of electric shock in fear conditioning paradigms (Sandi et al. 1992; Sandi et al. 1997). In contrast, memory facilitating effects of positive rewarding stimuli are less well studied. Using plain food as reinforcer, requires prior food deprivation of the subjects which is a stressor itself, known to change circadian corticosterone secretion and glucose levels (Makimura et al. 2003; Karami et al. 2006). Mice like sweets, so we decided to give the mice free access to glucose (sugar corns) as reinforcer.

It is well known that glucose facilitates cognitive performance and that peripheral glucose administration improves memory in aversive and appetitive tasks. In mice, glucose has always been administered via invasive techniques like intraperitoneal injections (Messier 2004). An intraperitoneal injection is an acute stressor, resulting in

increased heart rate, body temperature and elevated concentrations of corticosterone (Meijer et al. 2006; Dalm et al. 2008). As described above, stressors given in close context with a task have memory facilitating effects (Joels et al. 2006).

To dissect the rewarding properties of the post-training stimulus from interference with the stressful method of application, male C57BL/6J mice got free access to sugar in their home cage, either immediately post-training (0h) or 4h after the last training trial of the day. Separation of the rewarding stimulus in-time from the training event controls for general metabolic effects (Dawson and McGaugh 1971; McGaugh and Herz 1972). We expect that (1) post–training self-administration of glucose will reinforce memory resulting in superior cognitive performance and (2) the pattern of memory facilitation will be task-dependent.

Materials and Methods

Animals

Male C57BL/6J mice (3 months; n = 44) were purchased from Charles-River laboratories. Upon arrival at the animal facilities (Sylvius Laboratory, LACDR, University of Leiden, The Netherlands), mice were single housed and transported to the experimental room to acclimatize for two weeks before the start of the experiment, in a temperature (21 ± 1˚C) and humidity (55 ± 5%) controlled room; food and water *ad libitum;* 12-12h light-dark cycle (lights on at 0700h). All experiments were performed between 0900 and 1400h. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

Separate groups of mice were used in the two spatial learning tasks. Water maze (WM; n = 8/group): (1) post-training self administration of sugar in close context (0h-sugar), i.e., immediately upon return to their home cage; (2) post-training self administration of sugar out of context (4h-sugar), i.e. 4h after the last daily training trial in home cage as control for possible metabolic effects of sugar and (3) controls, i.e. no-sugar. The WM program started with a free swim trial, followed by 4 days of spatial training and finished with another free swim trial three days later. Circular Hole Board (CHB; n = 10/group): (1) post-training self administration of sugar in close context (0h-sugar) i.e. immediately

upon return to their home cage and (2) controls, i.e. no-sugar. The CHB program started with a free exploration trial, followed by four days of training and finished with another free exploration trial three days later.

Behavior was recorded on videotape and analyzed with Ethovision 1.97 (Noldus Information & Technology BV, Wageningen, The Netherlands). The software sampled the position of the mouse 5 times per second. To calculate the distance walked on the CHB, the minimal distance between samples was set at 3cm.

Self administration of sugar

Mice were familiarized with the sugar corns before WM and CHB training started. A feeding cup (2.5cm x 2.3cm) was glued to the bottom of the home cage in the corner opposite to the nest. During the week before training started, mice got free access to sugar three times (30mg sugar corns; every other day). At 0900h, the grid of the cage was lifted, the sawdust was removed from the feeding cup, and sugar was placed in the cup. Mice consumed all the sugar within 10 min. Mice remained in their home cage and were not handled during the administration procedure. Following the last training trial of the day, mice had free access to sugar in their home cage either immediately (0h-sugar) or delayed (4h-sugar), after having located the platform in the WM or exit tunnel in the CHB, or when the maximum trial duration had expired.

Water maze

Three days before spatial training started, the white pool (140cm diameter, side walls 50cm high) was filled with 2cm of warm water (26 ± 1°C). This was the mouse's first contact with water and it was allowed to walk around for 120s.

Training trials: The pool was filled with warm water (26 ± 1°C; ± 25cm deep) and made opaque by the addition of chalk. A platform (8cm diameter) was situated 0.5cm below the surface of the water, invisible for the mouse. The ratio between the surface area of the pool and the platform was 270:1. The mouse was placed in the water at one of four possible equally spaced release points. A maximum of 60s was allowed, during which the mouse had to find the platform and climb onto it. If the mouse did not find the platform itself it was guided there using a grid (20cm x 6cm). Mice remained on the platform for 15s. Animals were run sequentially with an inter-trial interval of approximately 10 min. After each trial, mice were placed under a red-light warming lamp for 3 min. A Free Swim Trial preceded and followed the spatial training trials (platform was absent: FST-before: 120s; FST-after: 60s).

Schedule and procedure: Day 1 started with FST-before, which allowed estimation of the swimming ability and to determine the pre-training exploratory strategy. One hour later, the first spatial training trial took place. On consecutive days, mice received four trials on days 2 and 3, followed by three training trials on days 4 and 5. Spatial training thus consisted of 15 trials over five consecutive days. Three days after the last spatial training trial, goal-directed search strategy was assessed in FST-after (day 8).

Spatial training trails were analyzed for: latency (s) and distance swum (m) to climb on the platform, swim speed (cm/s), cumulative distance to platform (m). To allow comparison, both free swim trials were analyzed for the first 60s. General activity was represented by total distance swum (m) and velocity (cm/s). Swim patterns were quantified on time spent in platform quadrant (percentage), latency (s), crossings (number) and cumulative distance to former platform location, relative to other possible 3 positions (Gallagher et al. 1993; Dalm et al. 2000). Thigmotaxis was expressed as time spent (%) close to the wall (RIM zone = 10cm).

Circular hole board

Apparatus: The Circular Hole Board (CHB) is a revolvable white Plexiglas plate (diameter: 110cm) with twelve holes (diameter: 5cm) at equal distance to each other, 10cm from the rim. It was situated 1m above the floor. In the original circular hole board setup (Barnes 1979) bright light and loud noise were used as aversive stimuli to motivate the animals to search for the exit. We performed the task under dim light conditions (120lux on the surface of the board), in a quiet surrounding and with numerous distal cues in the room which allowed spatial orientation. The holes on the CHB could be closed by a lid at a depth of 5cm. Whether a hole was open or not could be recognized by the mouse if it put its head over the edge of the hole. If open, the hole provided access to the home cage of the mouse via an s-shaped 15cm long tunnel (diameter: 5cm). Mice were `pre-trained' to climb through the tunnel three times every other day. This was performed in the week preceding familiarization to sugar corns, during weighing of the mice.

Training trials: Before each trial started, the board was swept clean with 1%HAc. Next, the board was turned clock- or anti-clockwise until the randomly determined hole was at the fixed location of the exit (spatial training). The home cage was placed underneath the exit tunnel (not visible for the mouse), and the mouse was placed in a non-transparent cylinder (PVC, diameter 10cm, height 25cm) at the center of the board. After 10s the cylinder was lifted and the mouse could explore the board. There was just one open hole during spatial training trials which was at the same location in all trials. As a control for possible odor cues, we turned and cleaned the board between trials, and

placed the home cage underneath the tunnel, opposite to the exit hole, during the Free Exploration Trial after training (FET-after). A free exploration trial preceded and followed the spatial training trials (all holes closed; FET-before: 300s; FET-after: 120s).

Schedule and procedure: Day 1 started with FET-before, which allowed to determine the pre-training exploratory strategy. After 5 min of exploration the animals were guided using a grid (20cm × 6cm), to the exit tunnel that they would need to search for during spatial training. Upon entering their home cage, they had free access to sugar (30mg). Spatial training was given on days 2 to 5: one exit hole was accessible in a fixed position. Mice received two trials per day with an inter-trial-interval of 15 min. If the mouse did not find the exit hole within 120s, it was guided there by a grid. Three days after the last training, FET-after (exit hole closed) was performed to determine whether spatial learning had altered the exploration into a goal-directed search strategy.

Spatial training trials were analyzed for latency (s), path length (m), velocity (cm/s) and time (s) before leaving the start area in the center (diameter 30cm). For the analysis of FET-before and FET-after, the CHB was divided into several zones of interest: (i) total arena: path length, velocity; (ii) start center, latency to leave center, percentage time spent; (iii) holes zone: latency hole area, hole visits, percentage time spent near exit and left/right adjacent hole; (iv) RIM zone: path length, velocity of moving, latency to RIM, percentage time spent. The latency (s) and path length (m) to the location of the hole used during spatial training were measured. The search strategies are described as *perseveration*: i.e. repeated visits of the same hole or alternately visiting two neighboring holes, and *serial*: i.e. more than two holes visited in sequence; calculated in relation to the total number of hole visits. A hole visit was detected if the animal had at least its nose over the rim of the hole. Detections by the image-analysis system were additionally cross-checked with manual protocols. To compare behavior during free exploration trials we analysed both trials 120s.

Statistical analysis

Data were subjected to ANOVA (factors: time, condition: 0h-sugar, 4h-sugar and no-sugar), when appropriate with repeated measures followed by a post-hoc Tukey test. Time in quadrants and platform crossings of the free swim trials were analysed with Friedmans Analysis of Variance (FR: per group) and Wilcoxon test (W: within group). Other parameters were compared with Student's T-test. We lost the data of the CHB 4h-sugar group due to computer problems. Data are presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Water maze: spatial training

All mice learned to locate the platform as indicated by a decrease in latency (Figure 1A) and path length (Figure 1B) to platform over days (latency: $F_{(4,84)}$=52.508, p = 0.001; distance: $F_{(4,84)}$=29.014, p = 0.001), with significant differences between the groups (latency: $F_{(2,21)}$=5.145, p = 0.015; distance: $F_{(2,21)}$=5.706; p = 0.01). Mice with post-training sugar administration in close context (0h-sugar) had the shortest latencies and distance to platform from day 4 onwards (compared to the 4h-sugar group on days 4, 5 and 8: p < 0.01; no-sugar group on days 4 and 8: p = 0.01). After day 3, the course of performance in latency and distance differed significantly between the groups ($F_{(8.84)}$=2.397, p = 0.022). While the latency and distance to platform continued to decrease in the 0h-sugar group, it remained at the same level in the other two groups. Swim speed remained constant

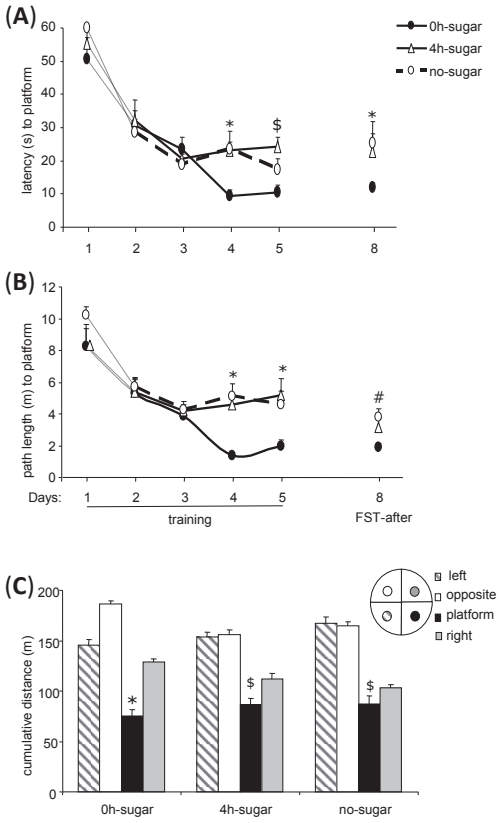

Figure 1

Water maze: (**A**) latency in seconds (s) and (**B**) distance swum in meters (m) to the platform during spatial training trials on day 1 (1 trial), days 2 and 3 (4 trials), days 4 and 5 (3 trials). For the free swim trial after training (FST-after; day 8), latency and distance are calculated based on the first visit of the former platform location. Mice consumed sugar in their home cage immediately after the last training trial of the day (0h-sugar) or 4h later (4h-sugar) or no-sugar. (**C**) Free swim trial *after* spatial training: Cumulative distance in meters to the former platform location (black bar) and virtual platform locations in adjacent and opposite quadrants (see inset). Less distance indicates more specificity towards the platform location. Data represent mean ± S.E.M.; **(A/B)** p < 0.05 * 0h-sugar vs. 4h- and no-sugar groups; $ 0h-sugar vs. 4h-sugar group; # 0h-sugar vs. no-sugar group. (**C**) p < 0.05 * platform location vs. the 3 virtual platform locations; $ vs. left and opposite virtual platform locations.

over the course of training and did not differ between the groups (data not shown). Interestingly, the latencies to platform of the first trial on days 4 and 5 were significantly lower in the 0h-sugar mice than in the 4h-sugar and no-sugar controls (Table 1). The performance in the other training trials of the day was variable. Performance in trials within one day (trial-to-trial performance) did not differ between the groups.

Water maze: Search strategies during free swim trials before and after spatial training

Before spatial training, mice of all groups behaved comparable regarding total distance swum, swim velocity and percentage time spent in RIM zone (Table 2A). After spatial training, on day 8, general activity between groups was again similar, but the 0h-sugar group was more active than before training (paired T-test: distance and velocity, p = 0.033). All groups spent less time in the RIM zone of the pool, indicating a shift in their swim strategy towards the open area of the pool where the platform was positioned during spatial training.

Spatial training altered the search strategy (Table 2B): Latency to the former platform location was shortest in 0h-sugar mice and their time spent in the platform quadrant was longer than in the other two groups. The number of platform crossings increased from FST-*before* to FST-*after* (paired T-test: $p < 0.05$), but did not differ between the groups. All groups directed their behavior towards the area of the platform location, but it was most specific for mice of the 0h-sugar group. They spent more time near the platform location, indicated by: (i) the lowest cumulative distance (Figure 1C; Friedman $p < 0.05$ vs. the virtual platform locations in the other three quadrants) and (ii) the increase in percentage of time spent in platform quadrant compared to FST-before (Friedman-Wilcoxon $p < 0.05$ vs. other quadrants). Also 4h-sugar mice had a significant lower cumulative distance to platform vs. the other three virtual locations. The no-sugar controls had a similar low cumulative distance to the platform and one virtual adjacent platform location, indicating less specificity of search patterns.

Circular hole board: spatial training trials

Latency and distance to the exit tunnel differed significantly between groups (Figure 2; main effect latency: $F_{(1,18)}$=19.652, p = 0.001) with significantly shorter latencies for the 0h-sugar group from days 2 to 5. In both groups, latency and path length decreased over days (latency: $F_{(3,54)}$=36.148, p = 0.001; distance $F_{(3,54)}$=4.053, p = 0.011), indicating learning of the task. Velocity of movement increased accordingly ($F_{(3,54)}$=20.689, p = 0.001).

Table 1: Latency to platform in seconds during the first trial of the day for the 0h-sugar, 4h-sugar and no-sugar groups

	Water maze: the first trial of the day			
	day 2	day 3	day 4	day 5
0h-sugar	48.9 ± 7.2	25.3 ± 9.1	9.3 ± 2.9*	6.7 ± 1.9*
4h-sugar	45.1 ± 7.2	24.9 ± 6.1	21.6 ± 6.3	16.9 ± 5.1
no-sugar	46.3 ± 9.0	29.0 ± 8.4	22.1 ± 7.7	18.8 ± 6.4

Data are presented as mean ± S.E.M.; * $p < 0.05$ vs. other groups, same day

Table 2a General activity expressed as path length swum, swim speed and percentage of time spent along the wall (RIM) in the water maze during the free swim trials *before* and *after* spatial training.

	0h-sugar		4h-sugar		no-sugar	
	before	after	before	after	before	after
path length (m)	9.3 ± 0.4	10.1 ± 0.4$^{\#}$	9.4 ± 0.5	10.4 ± 0.9	10.2 ± 0.4	11.1 ± 0.6
swim speed (cm/s)	15.6 ± 0.7	16.8 ± 0.9$^{\#}$	16.3 ± 0.9	17.4 ± 0.9	17.1 ± 0.7	18.6 ± 1.0
%time in RIM	59.9 ± 4.4	31.1 ± 3.8$^{\#}$	58.0 ± 2.7	21.1 ± 2.8$^{\#}$	65.2 ± 4.7	33.1 ± 8.3$^{\#}$

Data are presented as mean ± S.E.M.; $^{\#}$ $p < 0.05$ within group

Table 2b Free swim trial *after* spatial training: latency to and crossing of the former platform position; increase in percentage of time spent in the platform quadrant (free swim trial *before* = 100%).

	0h-sugar	4h-sugar	no-sugar
latency (s)	11.8 ± 1.7*	22.7 ± 5.6	25.3 ± 6.6
crossings	3.6 ± 0.4$^{\$}$	2.9 ± 0.6	2.1 ± 0.5
% time spent in platform quadrant	236.3 ± 22.0*	124.1 ± 23.3	182.6 ± 16.0

Data are presented as mean ± S.E.M.; $p < 0.05$ * vs. other groups; $^{\$}$ vs. no-sugar

Mice left the start area faster. This was group dependent (interaction time*condition: $F_{(3,54)}$=4.749, p = 0.005). On days 2 and 3 of training, mice of the 0h-sugar group had significantly shorter latencies to leave the start ($p < 0.001$) than no-sugar controls.

The slope and course of the learning curve for latency, path length and velocity over days was comparable between groups (interaction time*condition: latency, $F_{(3,54)}$=0.370, p = 0.774; distance, $F_{(3,54)}$=0.316, p = 0.814; velocity, $F_{(3,54)}$=1.494, p = 0.226).

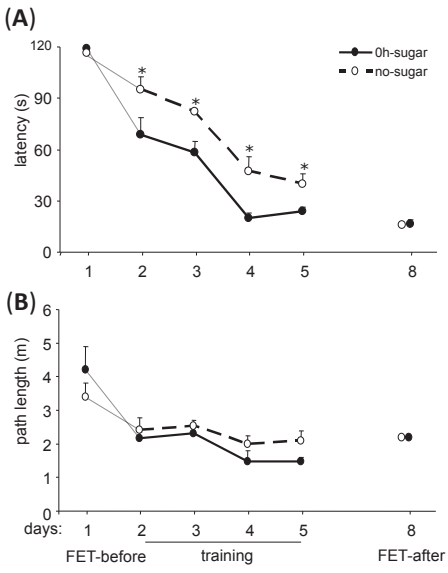

Figure 2

Circular hole board: (**A**) latency in seconds (s) and (**B**) distance walked in meters (m) to the exit hole during spatial training trials on days 2, 3, 4, 5 (2 trials per day) and during free exploration trials (FET) *before* (day1) and *after* (day 8) spatial training. Mice had received sugar in their home cage immediately after passing through the exit hole at the end of the FET-*before* and each day after the last training trial (0h-sugar) or no-sugar. Latency and path length during FET: FET-*before* indicates the distance walked during 120s; FET-*after* indicates the latency and distance to the first visit of the exit hole. Data represent mean ± S.E.M. $^{*}p < 0.05$ between groups.

Circular hole board: Spatial training trials

In addition to the mean daily performance, trial-to-trial performance within the day (short-term/working memory) revealed distinct differences. The first trial of the day of the 0h-sugar mice had the shortest latencies to the exit tunnel (Figure 3A; trials with odd numbers: trial 1, $p = 0.012$; trial 3, $p = 0.004$; trial 5, $p = 0.016$; trial 7, $p = 0.046$). The second trial of the day was always comparable to the no-sugar control group. In the 0h-sugar mice, time to leave the start center was significantly lower for trials 1 and 3 (all $p < 0.01$; Figure 3B). While mice of the 0h-sugar group have similar velocities in the first and second daily trial and keep their velocity constant from trial 1 to 7, no-sugar mice have lower velocity in trials 1 and 3 ($p < 0.05$; Figure 3C) and increase their velocity during their second trial of the day above the 0h-sugar mice ($p < 0.05$, trials 4 and 6). Path length was not significantly different between the trials (Figure 3D).

Circular hole board: General activity, exploration and search strategies

Before spatial training, the behavioral response, i.e., sum of analysed parameters, on the circular hole board was similar between groups (Table 3, MANOVA: $F_{(14,5)}=1.281$, $p = 0.420$). After spatial training, the behavioral response was not only different from before training, but also between groups (MANOVA: $F_{(14,5)}=6.635$, $p = 0.024$). Now, both groups were more active (increase in path length, velocity, total hole visits) and left the start

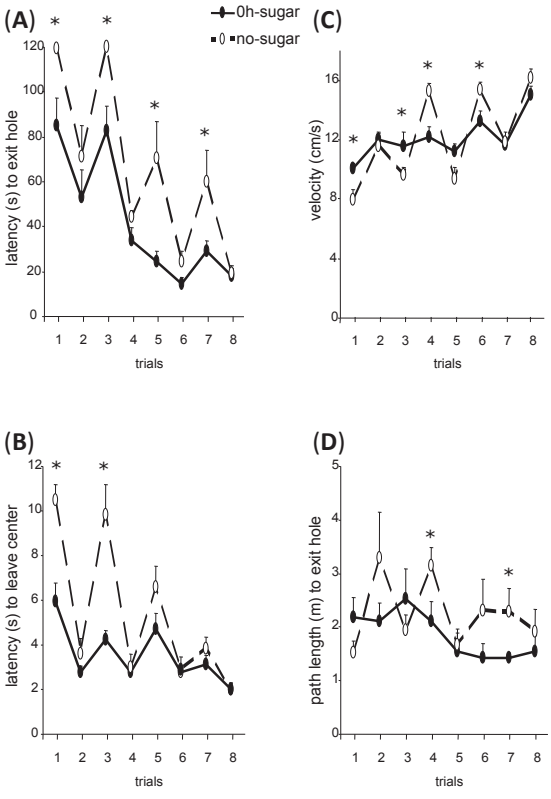

Figure 3

Circular hole board: Performance per trial during spatial training to locate the exit hole (days 2 – 5, i.e., trials 1 to 8) for mice that received sugar immediately after training (0h-sugar) or no-sugar. Odd numbers present the first trial of the day. (**A**) latency in seconds (s) to the exit hole, (**B**) latency to leave the center; (**C**) velocity in cm/s and (**D**) distance walked in meters (m) to the exit hole. Data represent mean ± S.E.M. * $p < 0.05$ between groups.

centre quicker resulting in shorter latencies to the hole and RIM zones (all $p < 0.05$). The 0h-sugar mice had the lower latencies to leave the start center ($p = 0.002$), to make the first hole visit ($p = 0.002$) and arrive at the RIM zone ($p = 0.040$). In both groups, the use of the perseveration strategy dropped dramatically from about 70% to 30%, while the use of the serial strategy increased from about 20% to 80% (both variables $p < 0.01$). In addition, time spent near the exit hole and adjacent holes increased specifically for the 0h-sugar group from FET-before to FET-after (208.7 ± 21.4%; paired T-test, $p = 0.001$) and was significantly higher than in no-sugar controls (134.9 ± 20.6%; $p = 0.023$).

Table 3 General activity parameters measured on the circular hole board during the free exploration trials *before* and *after* spatial training

		0h-sugar		no-sugar	
	Parameter	before	after	before	after
Total	Path length (m)	4.2 ± 0.7	7.7 ± 0.3#	3.4 ± 0.4	6.7 ± 0.5#
	Velocity (cm/s)	9.1 ± 0.7	13.2 ± 0.3#	7.3 ± 0.6	13.4 ± 0.5#
Center	Latency to leave center (s)	3.7 ± 0.5	1.7 ± 0.1*#	4.7 ± 0.4	4.3 ± 0.7
	% Time	3.5 ± 0.6	2.0 ± 0.3	5.0 ± 0.4	4.4 ± 0.9
Holes	Latency to hole area (s)	7.9 ± 0.7	3.4 ± 0.2*#	10.5 ± 1.7	6.3 ± 0.8#
	Hole visits (number)	12.6 ± 1.6	28.7 ± 1.6#	9.3 ± 1.1	23.2 ± 2.4#
	%time near exit and adjacent holes	100%	208.7 ± 21.4*#	100%	134.9 ± 20.6#
RIM	Path length (m)	0.9 ± 0.3	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1
	Velocity (cm/s)	7.0 ± 0.7	10.1 ± 0.6	5.4 ± 0.6	8.9 ± 0.9
	% Time	23. 8 ± 4.3	11.3 ± 1.4#	28.1 ± 5.0	12.8 ± 1.6#
	Latency (s)	23.0 ± 4.7	14.9 ± 2.9*	30.7 ± 8.0	27.0 ± 4.7
Search pattern	% Serial hole visits	20.6 ± 6.7	80.4 ± 4.1#	21.0 ± 6.5	80.6 ± 5.4#
	% Perseveration of hole visits	70.9 ± 4.4	30.9 ± 3.7#	63.8 ± 3.1	32.5 ± 5.6#

Data are presented as mean ± S.E.M.; $p < 0.05$, * between groups; # within group

Discussion

Post-training sugar reward facilitated the cognitive performance of mice in two spatial learning tasks: the Water Maze (WM) and the Circular Hole Board (CHB). The memory facilitating effects are expressed in a task-dependent pattern.

Post-training sugar reward and cognitive performance in the water maze and circular hole board

The WM and the CHB were originally designed for rats (Barnes 1979; Morris 1984). Studies comparing the behavior of rats and mice in the WM and CHB reported that the WM is less suited for testing spatial learning and memory in mice (Whishaw 1995; Whishaw and Tomie 1996; Wotjak 2004). Dry-land mazes like the CHB, take into account the predominant dry-land activity of mice and their aversion of water. Our data support that task-inherent properties differentially affect cognitive performance. For example, within-day performance largely varied for mice trained in the WM, while the exit hole was always faster located on the second trial of the day in the CHB task. Both tasks provide behavioral parameters related to general activity, and possible emotional and

motivational states. However, the CHB contributes more data for short-term memory, emotional and motivational processes (Grootendorst et al. 2001b) than the WM paradigm. Of course, the choice of the spatial learning task should be hypothesis driven. Modulation of consolidation was achieved by allowing mice free access to sugar in the home cage after the last training trial of the day. As expected, sugar reward in close-context with training (immediately, but not 4hrs later) facilitated memory in both spatial tasks, albeit within different time domains.

In the WM, the effect of sugar reward was expressed in latency and distance to platform from the fourth day of training onwards, i.e. after 12 trials, when 0h-sugar rewarded mice swam shorter distances to locate the exit platform during the first trial of the day. The superior performance was still expressed in the free swim trial three days following the last spatial training. These mice were more precise in navigating towards the previously learned location of the platform, spent most time around the former platform location, i.e. behavioral persistence. We may argue that memory for the platform location has been strengthened and/or it is less susceptible to extinction in the free swim trial. Swimming speed as indicator for increased motivation to reach the platform is less likely as it was comparable between groups. Out-of context rewarded mice (receiving sugar with a 4hrs delay) behaved more similar to no-sugar mice, further underlining the importance of close-context reward and its effect on consolidation. We conclude that post-training sugar in close-context results in improved performance via modulation of consolidation processes.

In the CHB task, memory improvement by sugar-reward was evident already on the first training day. How is this possible? The free exploration trial before training is actually the first sugar-rewarded trial. At the end of the free exploration trial, mice are guided to the exit hole, enter their home cage and get free access to sugar. The superior performance was maintained over the course of training. Whereas the learning curves for both groups run in parallel, sugar-rewarded mice reach their maximum performance on day 4, while control mice are still improving. Remarkably, sugar-rewarded mice had shorter latencies in the first trial of the day than control mice, while the second trial was comparable between groups. Parameters of the free exploration trial after training indicate that sugar-rewarded mice are more persistent in their search for the exit, spending more time in that area. We conclude that sugar in close-context to training affects long-term memory, but does not shift performance parameters in general. Mice of the no-sugar group require more training to reach a similar level of performance.

Emotion, motivation and memory

To differentiate effects on memory from motivational and emotional components, the CHB provides several parameters. For example, an increase in velocity to the exit hole might be indicative for motivational effects. Indeed, in the first trials on days 1 and 2, sugar-rewarded mice had shorter latencies to the exit holes and a higher velocity than no-sugar controls. However, on the following days short latencies remained in the face of comparable velocity in the first trial of the day. Moreover, in the second trial of the day velocity of sugar-rewarded mice was lower than in no-sugar controls. No-sugar mice moved faster on the second trial of the day. If velocity is an indicator for motivation, we have to consider a "trial-dependent" motivation that is apparent in the no-sugar control mice.

Spending more time in the central, most unprotected area is generally accepted as reduced anxiety-like behavior (Archer 1973; Choleris et al. 2001). On the CHB this will increase the latency to the exit hole. Indeed, no-sugar mice remained longer in the center during the first trial of the day. In the second trial, latency to leave the center was comparable between groups. There is no argument that receiving sugar the day before will change anxiety-related behavior. It is more likely that no-sugar control mice take more time for orientation, than being less anxious. Anyhow, the shorter time in center contributes to, but does not explain the shorter latencies to the exit hole in the sugar-rewarded mice. In relation to latencies and velocities, distance to exit hole indicates that sugar-rewarded mice move more goal directed than the no-sugar control mice.

We conclude that post-training sugar-reward in the CHB affects memory consolidation, most clearly expressed in the performance of the first trial of the day. Motivational and emotional aspects play a minor role.

Task-inherent activation of the stress system and glucose administration

Learning tasks present novelty to mice, with often rather aversive properties that activate the stress system, leading to the secretion of adrenal stress hormones: epinephrine and glucocorticoids. Facilitation of memory is a commonly reported effect, specifically when stress hormones are elevated in close-context with learning trials, i.e., during acquisition and specifically post-training (Gold 1986; De Kloet et al. 1998; McGaugh and Roozendaal 2002). Dose-dependent manipulation of corticosterone concentrations during and after training, either by lowering the water temperature or injecting the hormone, facilitates spatial learning in rats (Sandi et al. 1997; De Kloet et al. 1998; Akirav et al. 2004; Joels et al. 2006). In a parallel study using the same training protocols for WM and CHB, we

found corticosterone concentrations 20 min after the start of spatial training on day five to be higher in WM than in CHB trained mice (± 100ng/ml and ± 30ng/ml respectively; own unpublished data). This task-dependent corticosterone response might affect the slope of the learning curve in the WM and CHB task, interacting with the effect of sugar-reward.

Studies on the effect of sugar reward and other drugs on learning include handling, restraining and injecting the animal and thereby, additionally increasing stress-hormone secretion (Meijer et al. 2006). This task-independent activation of the stress system may contribute to the modulation of memory. Giving mice free access to sugar in close context with their performance in the learning task, we introduce a non-invasive method for sugar reward that is devoid of possible interfering effects of stress hormones on memory processes.

Reinforcement of behavior or reinforcement of a memory trace

Traditional reinforcement theory considers memory as something that is somehow determined by reinforcement and, thus, takes place *after* reinforcement. Reinforcers are thought to increase the probability of behavioral responses. This separation between theories of memory and theories of reinforcement, had been challenged by Huston and colleagues (Huston et al. 1974; Huston and Mondadori 1977; Huston and Oitzl 1989) proposing an integrated theory of memory and reinforcement. After the performance of a learning task (i.e., during the post-trial, post-training period) memory remains susceptible to disruptive or facilitating treatments. Memory is still in a labile form prior to being fixed or consolidated in a more permanent form (McGaugh and Herz 1972). Consequently, positive reinforcers (reward; for a discussion on the difference between reward and reinforcement: see (White 1989) presented after the learning trial during periods of labile memory should also promote learning. In their first study (Huston et al. 1974), mice received an aversive electric footshock when stepping down from a platform. Should the reinforcer facilitate the behavioral response, mice are expected to step-down faster in the test trial. On the contrary, post-trial presentation of food facilitated inhibitory avoidance learning: the animals remained on the platform longer than controls. This finding and a series of studies using other aversive, but also appetitive tasks (summarized in (Huston and Oitzl 1989), support the theory that the reinforcer (food, electrical brain stimulation, substance P) acts on the central consequences of behavior, i.e. a memory trace; and not the behavioral response itself.

In the present study, mice had access to sugar after the last training trial of the day. Long-term memory is improved by sugar-reward in both spatial tasks, expressed

as superior performance in the first trial of the following day. Whereas the memory facilitating effect in the CHB is observed already after the first contingency: location of and moving through the exit hole and sugar consumption, it takes several days until it is obvious in the WM. As suggested before, this time-related effect of the reinforcer is most likely due to task-inherent properties. However, common to both tasks is that goal-directed behavior during training trials and the persistence of the search pattern in the area of the platform and exit hole are strengthened. General activity and velocity as behavioral responses to the task are not reinforced. Thus, it is the memory trace of: how to locate the platform or exit hole that is strengthened by sugar reward. The memory facilitating effects of sugar are most obvious in the earlier phases of learning. We conclude that our findings substantiate the theory of an integrated reinforcement and memory process.

Conclusion

Post-training sugar facilitates spatial memory in mice. The pattern of the memory facilitating effects depends on the task-inherent properties of the WM and CHB. In line with others (Whishaw 1995; Wotjak 2004), we consider the CHB better adapted to the species-specific needs of mice. Moreover, it allows to collect a broader set of variables related to motivation and emotional expression than the present WM paradigm. The limited number of training trials in the CHB task gives way to pharmacological interventions in close context with training events. The non-invasive administration method of sugar discarded the generally adverse effects related to the method of treatment. Post-training self-administration of sugar proved to be an exciting approach to reveal the effects of reinforcers on the formation of memories. Since changes in the reward processing system belong to the main symptoms of stress-related diseases like depression (e.g., anhedonia), we propose that our test-paradigm will be a valid tool to test reinforcement processes in animal models of such stress-related diseases.

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Chapter 6

Repeated rat exposure inhibits the circadian activity patterns of C57BL/6J mice in the home cage

Sergiu Dalm*
Leonie de Visser*
Berry M. Spruijt
Melly S. Oitzl

*authors contributed equally to the manuscript

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Abstract

Exposing male C57BL/6J mice repeatedly, in an unpredictable and uncontrollable fashion to rats, alters their cognitive performance and the neuroendocrine stress response, weeks to months after the rat stress. Continuous observation of the behavioral activity of male C57BL/6J mice in their home cage before (baseline) and after rat exposure could reveal if repeated rat exposure leads to changes in circadian activity patterns, which is a key feature of chronic stress and stress-related disorders in humans.

Rat stress (1) decreased exploratory and foraging activity as characterized by increased time spent in the shelter and less time in the open area; (2) reduced sucrose consumption and inhibited the development of sucrose preference, suggesting changes in the reward system and (3) the exploration pattern in a novel environment included more behavioral perseverations, but no change in general locomotor activity. Comparison to baseline activity pattern, i.e. before any intervention, revealed that already the control procedure to rat exposure (spending the same amount of time in another cage) disrupted the organization of behavioral activity patterns, albeit to a different and lesser degree than observed in rat stressed mice.

While only the longitudinal design of the study allowed detecting these dynamic patterns of circadian activities, the distinct behavioral changes in foraging and explorative activities support our notion that repeated rat exposure might serve as mouse model of chronic stress.

Introduction

Chronic stress, specifically a dysregulation of the glucocorticoid system, is thought to be a precipitating factor in the etiology of affective disorders (de Kloet et al. 2005). These disorders share several characteristics: emotional changes related to approach/avoidance behavior, loss of interest or pleasure in daily activities, impairment of cognitive functions, reduced motor activity and alterations in the circadian pattern of physiological, neuroendocrine and behavioral responses (Endo and Shiraki 2000; Volkers et al. 2002; Keller et al. 2006). The effects of chronic stress in animal models are mainly assessed in short-lasting test-situations involving additional novelty stress. Surprisingly little is known about the consequences of stress on the daily organization of behavior in a familiar environment where the animal spends most of its time: the home cage. This will be the focus of the present study.

Whereas all kinds of stressors induce behavioral alterations and concomitant changes in stress system regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Endo and Shiraki 2000; Anisman and Matheson 2005), psychological stressors are ethologically relevant and resemble the kind of stress that is related to affective disorders in humans (Calvo-Torrent et al. 1999; Apfelbach et al. 2005; Beekman et al. 2005). Central features of chronic psychological stressors in humans are repeated, unpredictable and uncontrollable exposure to (or imagination of) threatening situations. Animal models make use of confrontations with territorial conspecifics and exposure to predators with or without physical confrontation (Apfelbach et al. 2005). Interestingly, already sensory stimuli (visual, auditory and olfactory) appear to be sufficient to activate the stress system associated with the release of glucocorticoids (Blanchard et al. 1998; Diamond et al. 1999; Linthorst et al. 2000a; Beekman et al. 2005). In rodents, the behavioral effect of predator exposure is manifested as increased anxiety-like behavior, risk-assessment in novel environments and learning and memory impairments (Calvo-Torrent et al. 1999; Grootendorst et al. 2001a; Grootendorst et al. 2001b; Adamec et al. 2004; Diamond et al. 2006). The amplitude of circadian locomotor activity and food-intake in rats decreases after social conflict, “chronic mild stress” or electric shocks (Willner 1984; Desan et al. 1988; Stewart et al. 1990; Gorka et al. 1996; Meerlo et al. 1999). Resident/intruder pairs of mice living in continuous sensory contact and daily physically interaction reveal a variety of changes in behavior, autonomic and immune functions, HPA responses, brain cytokine expression and cardiac histology (Bartolomucci et al. 2005). To our knowledge, detailed patterns of activity in the home cage before, during and after a psychological stressor, without physical contact, have not been described in mice.

Previous studies have shown that, long term automatic recordings of the location of the mice in their home cage allows detailed observations on dynamic changes in locomotor activity over days, with minimal human intervention (de Visser et al. 2005; de Visser et al. 2006). In addition, the home cage is a familiar environment in which no specific behavior of the animal is elicited or challenged in some way. Subtle changes in spontaneous behaviors under base line conditions may reveal themselves more easily in the home cage than under conditions where the animal is prompted to explore or face a strong challenge.

The aim of the present study was to investigate the daily behavioral organization of mice in the familiar environment of their home cage: before, during and after chronic 'rat stress' exposure, which took place in a novel environment.

Our chronic stress model makes use of the observation that mice and rats avoid each other in nature. Indeed, repeated, unpredictable and uncontrollable exposure of mice to rats strongly activates the HPA axis (Grootendorst et al. 2001a; Grootendorst et al. 2001b). To control the effect of rat exposure on HPA axis activation, blood plasma corticosterone concentrations were measured before and after the first rat exposure. Furthermore, we determined consumption of and preference for a sucrose solution as markers for altered consumatory behavior and anhedonic consequences of stress. To address behavioral changes to novelty, exploration patterns of mice in a novel environment (circular hole board) were assessed two days after the last rat exposure. Principal Component Analysis (PCA) was performed to determine the relationship between activity parameters in the home cage which may be indicative of underlying motivational systems.

Materials and Methods

Animals

Upon arrival at the animal facilities of Utrecht, male C57BL/6J mice (Janvier Bioservices, The Netherlands; n = 32; 8 weeks) were individually housed with food and water *ad libitum* in Macrolon Type II cages for one week. The room was temperature (19 - 21°C) and humidity (30 - 50%) controlled with a 12-12h light-dark cycle (lights on 0800-2000h). A shelter and nesting material (tissues, paper shreds) were provided. Thereafter, mice were housed individually in PhenoTyper cages. Male Long Evans hooded rats (Janvier, n = 8; 250 - 275g) were housed in pairs in a separate room, with food and water *ad libitum*. Experiments were approved by the Local Committee for Animal Health, Ethics

and Research of the Universities of Leiden and Utrecht. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC) and the Principles of laboratory animal care (NIH publication No. 86-23, revised 1985).

Experimental design

Figure 1 depicts an overview of the experimental schedule. Mice were housed for one week in Macrolon Type II cages (days -6 to 0). These cages were kept with the original bedding but without nesting material, for rat stress and control procedures. Automated registration of activity and location of the mice took place in the PhenoTyper home cages every day for 24h from days 1 to 19, and was interrupted only by experimental procedures. Mice were subjected to two conditions (n = 16/condition); (i) stress: exposure to rats during 2 weeks and (ii) control: placement into their first housing Macrolon Type II cages at similar times and duration as the rat stress condition. On day 6, blood samples were collected via tail incision before and directly after the first rat exposure. Sucrose solutions were available for 24h on days 5, 13 and 17. The exploration strategy of a

Day		Experimental manipulation
-6 – 0		Housing in Macrolon Type II cages
1	Housing in PhenoTyper	Baseline bodyweight
2		
3		
4		Baseline home cage activity and location
5		Baseline sucrose consumption/preference
6		Rat exposures 1 and 2
7		Rat exposures 3 and 4
8		Rat exposures 5 and 6
9		Rat exposure 7
10		Rat exposures 8 and 9
11		
12		
13		Sucrose consumption/preference
14		Rat exposure 10
15		
16		Rat exposure 11
17		Sucrose consumption/preference
18		Exploration on the circular hole board, 5 min
19		

Figure 1
Time line of the experiment. Data of home cage activities are presented from several days, shaded in gray (see Figures 4, 5 and 6). Black squares at day 6 indicate time of blood sampling for determination of corticosterone concentrations.

novel environment (the circular hole board), was assessed on day 18. Bodyweight was measured from the day of arrival until the end of the experiment on a daily basis.

Home cage behavior

Apparatus - Home cage behavior was automatically recorded by videotracking in specially designed cages for automated recordings (PhenoTyper®, Noldus Information Technology, Wageningen, The Netherlands, see Figure 2A). Each cage (30cm x 30cm x 35cm) was equipped with a feeding station and two drinking bottles. A shelter (10cm x 10cm x 5cm), bedding (sawdust) and nesting material (tissues) were provided. Hardware for videotracking is integrated in a unit on top of the cage, which also contains a built-in digital infrared-sensitive video camera and infrared lighting sources. The infrared sources provide a constant and even illumination of the cage to allow videotracking irrespective of light conditions in the experimental room.

Videotracking - EthoVision 3.1 (Noldus IT, The Netherlands) was used as videotracking software. Within EthoVision, we designed several zones per cage to extract behavioral measures of duration and frequencies of visits per zone (see Figure 2B): shelter, feeder, bottles 1 and 2. The open area covers the rest of the surface. The shelter was defined as a "hidden zone" allowing the program to distinguish the location of the mouse as being "in the shelter" or "on the shelter".

Videotracking was performed with the maximal sample rate of 12.5 samples/second. The system is programmed to score changes in the location of the centre of gravity of the mouse as "movement", only when the mouse moved at least with a velocity of 3.5cm/second, averaged over 12 samples. This excludes small movements of

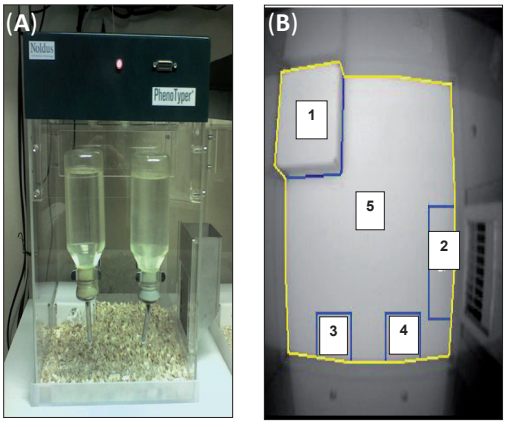

Figure 2
(A) Picture of the automated home cage observation system with two drinking bottles, shelter and feeding area. **(B)** Schematic overview of the different zones that were used for analysis of home cage behavior: 1=shelter; 2=feeder; 3=bottle 1; 4=bottle 2; 5=open area.

the animal caused by e.g., turning or moving around while staying in the same place. The number of stops was calculated using the frequency of 'non-movement' fragments, per unit distance movement. This yielded a measure that is independent of overall amount of activity (de Visser et al. 2006). Velocity was calculated only of 'movement' episodes, thus excluding periods of non-movement.

Dependent variables - Per zone, several parameters were calculated which were subjected to further analysis: duration (time spent in a specific zone), frequency (number of visits to and from a specific zone), cage floor movement (time spent moving in the open area in seconds), distance moved (distance travelled by the animal in the open area in cm), velocity (speed of moving in cm/s) and the number of stops (periods of non-movement per unit distance moved). All parameters were calculated in 1-hour intervals and subsequently summed for the 12-hours fragments of the dark and light period. For the nocturnal pattern of activity, we used the hourly values of the parameter cage floor movement during the dark period.

Rat stress paradigm

Exposure to a rat profoundly activates the Hypothalamic-Pituitary-Adrenal (HPA) axis of the mouse, resulting in elevated concentrations of corticosterone in brain lysate (Linthorst et al. 2000) and blood plasma (Grootendorst et al. 2001a). We have designed the "mouse-exposed to rat" procedure some years ago (Grootendorst et al. 2001a; Grootendorst et al. 2001b), taking into account central features of a stressor: unpredictability, uncontrollability. The protocol uses repeated exposure to rats (i) either daily or with a break of one or several days; (ii) varying the duration and time of the rat exposure: 1 or 2h; once or twice a day; (iii) each time placing different rats on top of the mouse cage – and thus avoiding physical contact that might involve pain.

Several times between days 6 - 16, mice were exposed to a rat (Figure 1). During the first week (days 6 - 10) of the rat stress paradigm, mice were exposed to rats on 5 consecutive days (one or two hours a day resulting in a total exposure time of nine hours). In the second week (days 13 - 16), two exposures took place: on Tuesday (1h) and Thursday (1h). On rat-exposure-days, mice were placed and transported in their first Macrolon Type II housing cages from the PhenoTyper to the adjacent rat stress room. One rat was placed on top of two mouse cages. Mice and rats were separated by a grid and could see, hear and smell, but not touch each other. Food and water was not available during rat exposure. The person who performed the rat stress procedure did not enter the housing room of the mice, as to avoid confrontation of control mice with the smell of rats. To keep time and duration of exposure unpredictable for the mice, the

exposures took place at different times during the light period, lasting one or two hours, either once or twice a day. The location of the cages and the combination of the rat and the mouse were changed *ad random*. Rat stress thus consisted of transportation and the exposure of mice to rats. Control mice were placed into their previous Macrolon Type II cage for the same duration as mice exposed to rats, but were not transported to another room. Thus, in both conditions, mice were removed from the PhenoTyper home cage. To asses the effect of rat exposure on arousal, mice were weighed before and directly after the last rat exposure of the day. Weight loss was taken as a measure of defecation and urination. Similar time points were used for control mice.

Sucrose consumption

Throughout the experiment two drinking bottles were fixed to the home cage. To determine if mouse preferred to drink from one of the two water bottles, the bottles were weighed on days 1 and 4. During sucrose consumption/preference testing, water from the least preferred bottle was replaced with a 5% sucrose solution. Consumption of water and sucrose was determined one day before (baseline; day 5), two days after the ninth rat exposure (day 13) and one day after the last rat exposure (day 17). The bottles were weighed before placement and after removal 24h later. Water and sucrose consumption is expressed in ml. The preference for sucrose was calculated as percentage of consumed sucrose solution of the total amount of water and sucrose consumption. After sucrose testing, both bottles contained water.

Blood sampling and corticosterone measurement

To determine the level of stress system activation in response to experimental procedures, blood was collected via tail incision (Dalm et al. 2005) before and after one hour of rat exposure (stress group) or placement in the Macrolon Type II home cage, on day 6. Blood was collected individually in capillaries (coated with potassium-EDTA, Sarstedt, Germany), kept on ice and centrifuged for 10 minutes with 13000 rpm at 4°C. Blood plasma was stored at –20°C. Plasma corticosterone was analyzed using a commercial available radio immunoassay kit ^{125}I-corticosterone (MP Biomedicals CA; USA; sensitivity 3ng/ml).

Circular hole board

Apparatus: A grey round plate (Plexiglass; 110cm diameter) with 12 holes (5cm diameter, 5cm deep), at equal distances from each other and at a distance of 10cm

from the rim of the hole to the rim of the plate, was situated 1m above the floor in a different experimental room. Light conditions on the surface of the board were 120lux. To distribute odor cues, the surface was cleaned with 1%HAc and the board was turned (randomly clock- and anticlockwise) before a mouse was tested.

At day 18, two days after the last rat exposure, mice were taken from their home cage, transported in a Macrolon Type II cage, picked from the cage at the base of the tail, and placed in a grey cylinder (PVC, 10cm diameter; 25cm high) that was located in the center of the circular hole board. After 10s the cylinder was lifted and the mouse could start to explore the board for 5 min. Immediately thereafter, the mouse was transported back to the PhenoTyper home cage. Behavior was recorded on videotape and analyzed by EthoVision Windows 3.1 (Noldus Information and Technology BV, Wageningen, The Netherlands). The image analysis system sampled the position of the mouse 12.5 samples/second. To calculate the distance moved, we set the system to score movement when the mouse moved at least with a velocity of 3.5cm/second, averaged over 12 samples. The following parameters related to general activity, exploratory strategies and possible anxiety-related behaviors were analyzed: distance walked (m) on the board and in specified zones: center, rim; velocity (cm/s), number of holes visited; sequence of hole visits (*serial*: more than two hole ins sequence; *perseveration*: repeatedly visiting the same hole or alternately visiting two neighbouring holes); latency (s) to leave the center; latency (s) to rim; time (s) in specified zones. The center is defined as a circle of 30cm diameter; hole area: a ring of 15cm with the holes in the middle; rim area: a ring of 4.5cm at the outer perimeter of the plate.

Additional experiment on sucrose consumption and novel cage in non-stressed mice

It has been shown that HPA axis activity and sucrose consumption are linked (Bell et al. 2000; Laugero et al. 2001). To assess if sucrose consumption itself might affect locomotor activity in the dark period, we performed an additional experiment. We used a separate set of male C57BL/6J mice (n = 16) to assess the effect of sucrose consumption on activity patterns in the home cage. Some mice were also exposed for two hours to their previous housing cage. Mice were initially housed individually in Macrolon Type II cages for one week (lights off from 1400 to 0200h; these cages, including the soiled bedding were kept and used for "novel cage" exposure later on). Thereafter, mice were housed in the PhenoTyper home cages for 5 days, and assigned to three groups: (1) *controls* (n=4) undisturbed from days 5 - 8; (2) *sucrose* (n=6) received a bottle with sucrose solution on day 5 at 0900h for 24h and not disturbed on days 6, 7 and 8; (3) *sucrose+NovelCage* (n

= 6) also received a bottle with sucrose solution on day 5 for 24h; on day 6, mice were placed in their previous Macrolon Type II cage (from 0900 - 1000h and 1300 - 1400h), and left undisturbed thereafter on days 7 and 8.

Statistical analysis

Home cage behavior: During the light period, activity was minimal and did not show any differences between groups or experimental days. Therefore, we limit the analysis to the dark period; i.e., the active period of mice, and selected the following experimental days (see Figure 1): day 4 (baseline), day 6 (after two exposures), day 10 (after nine exposures), day 14 (after 24hrs sucrose consumption and ten exposures) and day 19 (three days after the last exposure). Differences between control and stress groups for each day were determined using a *t*-test; within groups between days were tested by a paired samples *t*-test with a Bonferroni-correction for the number of comparisons. We selected to present the following parameters: time spent in the shelter, near the feeder and bottles and the distance moved in the open area, in Figure 4A-D. To test for differences between groups and days in the diurnal pattern of cage floor movement, repeated measures ANOVA was performed, using the within-subjects factors “hour”, for the 12 hours of the dark period, and “day”, for the four experimental days (6, 10, 14 and 17), and the between-subjects factor “group”, for control and stress groups. Post-hoc comparisons between days were done using repeated measures ANOVA with within-factors “hour” and “day”.

To investigate the interrelation of dependent variables measured in the home cage and to identify possible independent factors, we performed a Principal Component Analysis (PCA) with varimax rotation (Ferguson 1981; de Visser et al. 2006).Dependent variables that showed a loading >0.6 were regarded as being relevant for a specific factor. Factors with Eigenvalues >1 were retained for further analysis. To determine the factors, PCA was performed across individuals for baseline (day 4), thus before any manipulation had taken place. Then, based on this factor structure, factor scores were calculated for each animal on each day by multiplying the mean values of each 12-hour bin with the factor loadings to create new dependent variables. These new variables thus consist of the combined values of the dependent variables that belong to a given factor. These variables were further analyzed with a *t*-test to detect differences between control and stress groups and with a paired samples t-test to test for within-group differences between days. A Bonferroni-correction was used when multiple comparisons were made.

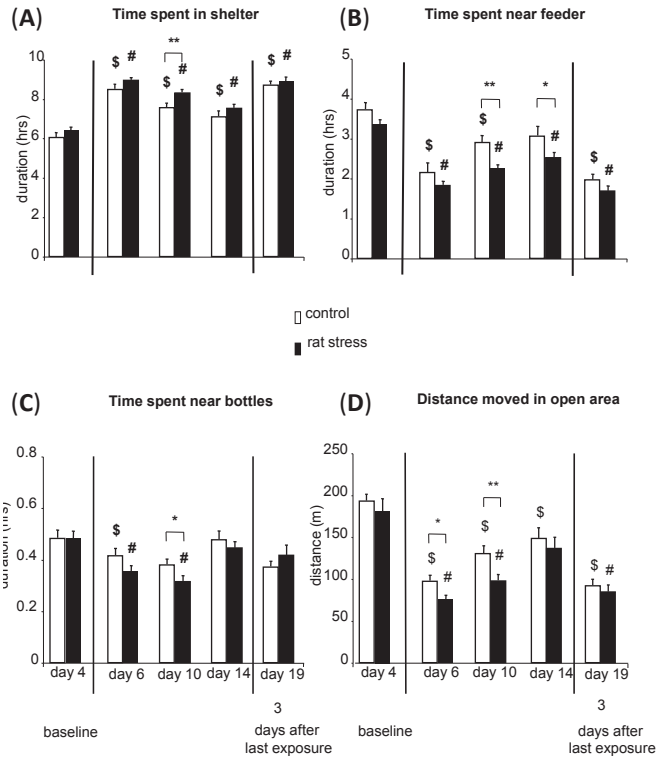

Figure 3

Home cage activity during the 12hrs lasting dark period: control (white bars) and stressed mice (black bars) on day 4 (baseline: before experimental manipulations start), day 6 (after two exposures), day 10 (after nine exposures), day 14 (after 24hrs sucrose consumption and ten exposures) and day 19 (three days after the last exposure and one day after circular hole board exploration). See Figure 1 for details on the experimental schedule. Home cage activity is represented by four dependent variables: **(A)** time in shelter, **(B)** time spent near the feeder, **(C)** time spent near the bottles and **(D)** distance moved in the open area. Data is presented as mean ± S.E.M. of the 12hrs dark period. Symbols indicate significant differences from baseline for control ($^{\$}$) and rat stress ($^{\#}$): paired samples t-test with Bonferroni correction $p < 0.05$; asterisks indicate significant differences between groups per experimental day: * $p < 0.05$, ** $p < 0.01$ (t-test).

Data on bodyweight, blood plasma corticosterone, sucrose consumption and preference, are presented as mean ± S.E.M.; tested using repeated measures ANOVA, and post-hoc t-test when appropriate. Depending on the normality score obtained with Kolmogorov-Smirnov, parameters of circular hole board behavior were compared using

one way ANOVA or Mann-Whitney test. Data is presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Home cage behavior

Mice were repeatedly exposed to rats (rat stress group) or placed in their previous housing cage in the same time schedule (control group). Rat stress had a distinctive influence on behavior of mice in the home cage (Figure 3A-D). These mice were less active than controls during the dark period. They spent less time at the feeder and the bottles and moved less (days 10 and 14, T-test, $p < 0.05$), but spent significantly more time in the shelter (day 10: T-test, $p < 0.05$). Three days after termination of the stress procedure, home cage behavior was comparable between control and stress groups for the parameters tested.

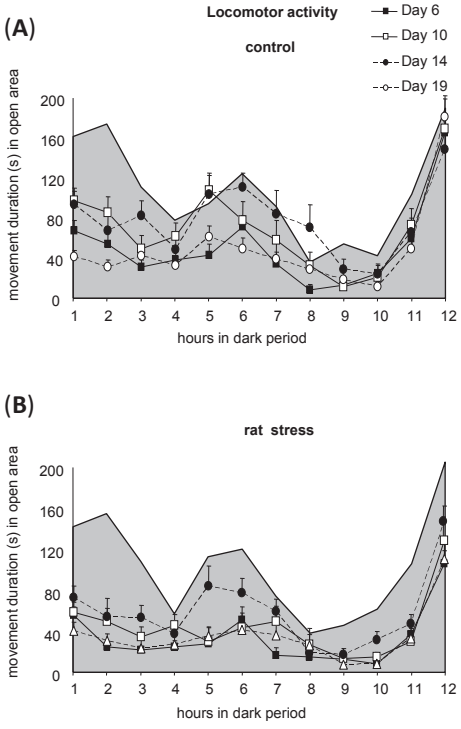

Figure 4

Hourly distribution of locomotor activity (cage floor movement), **(A)** control and **(B)** rat stress group, expressed as the time spent moving in the open area in seconds per hour (mean ± S.E.M.) during the dark period of day 4 (baseline), day 6 (after 2 exposures), day 10 (after 9 exposures), day 14 (after 10 exposures) and day 19 (three days after exposure). See Figure 1 for details on the experimental design. Shaded area represents mean activity on day 4.

Interestingly, also the control manipulation changed the level of nocturnal activity. Baseline measurements from day 4 allowed within-group analysis over days for control and stressed mice. Independent of group, significant differences from baseline were found for the time the mice spent in shelter (Figure 3A), near to the feeder (Figure 3B), near the drinking bottles (Figure 3C) and the distance moved in the open area (Figure 3D). For all days analyzed, activity in the open area as well as activity at the feeding and drinking places was lower compared to baseline (all days, paired samples T-test, $p < 0.05$). The drop in activity was largest in response to the first experimental manipulations in control and stressed groups (day 6), followed by a gradual increase towards baseline levels from days 10 to 14. Notably, three days after termination of the stress procedure on day 19, activity was comparable to levels observed on day 6: again, more time in shelter, less near the feeder and bottle area and reduced locomotion.

Nocturnal pattern of cage floor movement

The nocturnal pattern of cage floor movement is presented per hour for control (Figure 4A) and stressed mice (Figure 4B). As activity during the light period was minimal and did not show any group difference, it was decided to exclude these data from Figure 4A-B. Characteristic for the nocturnal pattern at baseline are the peaks in activity at the beginning and end of the dark period. Compared to baseline, both groups showed a significant change in the activity pattern (repeated measures ANOVA, interaction between within-subjects factors “day” and “hour”, $F_{(44,100)}=2.471$, $p = 0.003$). Post-hoc analysis revealed significant differences from baseline for all days (day 6, 10, 14 and 19; $p < 0.01$). The difference was most pronounced during the first 3 hours of the dark period, expressed by the markedly reduced peak during and after the experimental manipulations.

Principal Component Analysis of home cage behavior

The interrelation of variables measured in the home cage is represented by three factors, extracted by Principal Component Analysis (Table 1). The factors together accounted for 70.59% of the variance. Factor 1 loads positively on variables that indicate exploration, like time spent on the shelter and distance moved in the open area. Factor 1 is labelled “exploratory activity”. Factor 2 consists of variables that indicate the activity directed towards the feeding and drinking areas, like time spent near the feeder and the bottles: Factor 2 is labelled “foraging activity”. The third factor consists of variables like time spent in the open area, velocity and the number of stops suggesting a specific aspect of

Table 1: Principal Component Analysis

Variable	Factor 1	Factor 2	Factor 3
In shelter (frequency)	.691		
On shelter (frequency)	.791		
On shelter (duration)	.819		
Distance moved in open area	.920		
Cage floor movement	.881		
In shelter (duration)		-.898	
Feeder (duration)		.841	
Bottles (frequency)		.654	
Bottles (duration)		.775	
Open area (duration)			.690
Velocity			-.893
Number of stops			.619
% of variance explained	*31.50*	*22.90*	*16.19*

movement in the open area that is independent of general activity: Factor 3 is labelled "velocity/stops".

To investigate the effects of the stress paradigm on the individual scores of each of the factors, the factor scores of each mouse were calculated for the dark period of days 6, 10, 14 and 19. Figure 5 presents the data for Factors 1 and 2; Factor 3: "velocity/stops" was comparable between groups (data not shown).

Factor 1: "exploratory activity" was significantly decreased with respect to baseline on days 4 and 19 in control and stressed mice (paired samples T-test, $p < 0.05$). Stressed mice showed a further decrease compared to control mice during the stress procedure (days 6: t = 2.399, df = 30, $p = 0.023$; day 10: t = 2.355, df = 30, $p = 0.025$), but not after termination of the stress procedure (day 19). For Factor 2:"foraging activity", a decrease was found on all experimental days compared to baseline, both in control and stressed mice (paired samples T-test, $p < 0.05$). Furthermore, stressed mice scored less than controls during the stress procedure (days 6: t = 2.355, df = 30, $p = 0.025$; day 10: t = 4.581, df = 30, $p < 0.001$; day 14: t = 2.233, df = 30, $p = 0.033$). After termination of the stress procedure (day 19) factor scores were comparable between control and stressed mice.

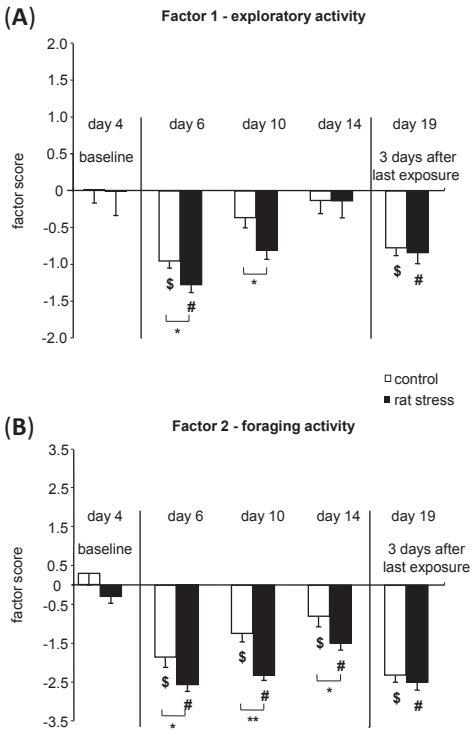

Figure 5

Home cage activity represented by two factors extracted from Principal Component Analysis (PCA); **(A)** Factor 1 indicates "exploratory activity"; **(B)** Factor 2 indicates "foraging activity". Factor scores were calculated for every mouse using factor loadings derived from PCA (see Table 1). Means per group for control (white bars) and rat stress (black bars) are presented for day 4 (baseline), day 6 (after two rat exposures), day 10 (after nine rat exposures), day 14 (after ten rat exposures) and day 19 (three days after the last rat exposure). See Figure 1 for details on the experimental design. Note that factor scores are relative measures and do not represent a specific measured variable. Data is presented as mean ± S.E.M. of the 12hrs dark period.

Symbol ($^{\$}$) indicate significant differences from baseline both groups (paired samples t-test with Bonferroni correction $p < 0.05$) asterisks (*) indicate significant differences between groups per experimental day: * $p < 0.05$, ** $p < 0.01$ (t-test).

In summary, removal from the home cage decreases both exploratory and foraging activity in control and stressed mice. However, there is a marked additive decreasing effect of rat exposure on exploratory activity and, more pronounced, on foraging activity.

Sucrose and water consumption and preference

Data are presented in Table 2. Fluid consumption on day 5, the day before rat stress began, was taken as baseline (100%): sucrose 12.7 ± 0.6ml and water 2.4 ± 0.1ml. The pattern of sucrose intake changed over time (time: $F_{(1,30)}$=47.646, p = 0.001) and was group dependent (group: $F_{(1,30)}$=24.896, p = 0.001). While sucrose intake was increased in both groups (paired samples T-test: $p < 0.05$) from day 13 to 17, this increase was significantly higher in controls than stressed mice, 42.2 vs. 17.7%, respectively. On both

Table 2: Sucrose and water consumption / preference in relation to baseline values. Baseline fluid intake: sucrose 12.7 ± 0.6 ml; water 2.4 ± 0.1 ml.

		Day 13 Two days after 9 exposures		Day 17 One day after 11 exposures	
	Fluid	% intake (ml)	preference (%)	% intake (ml)	preference (%)
Control	Sucrose	102.5 ± 4.8	88.7 ± 0.4	144.2 ± 5.3	91.3 ± 0.3
	Water	76.4 ± 3.7	11.2 ± 0.2	79.6 ± 4.9	8.7 ± 0.7
Rat stress	Sucrose	87.0 ± 5.8*	87.0 ± 0.8	104.7 ± 4.9*	87.2 ± 0.2*
	Water	68.1 ± 1.7*	12.9 ± 0.5	88.2 ± 3	11.9 ± 0.1

Data are expressed as mean ± S.E.M.

* $p < 0.05$ rat stress vs. controls.

testing days, stressed mice consumed less sucrose than controls (one way ANOVA, day 13: $F_{(1,30)}$=5.552, p = 0.025; day 17: $F_{(1,31)}$=38.188, p = 0.001). The pattern of water intake also changed over time (time: $F_{(1,30)}$=25.926, p = 0.001) and differed between groups (group: p = 0.001). Stressed mice had a significantly reduced water intake on day 13 (one way ANOVA, $F_{(1,31)}$=4.192, p = 0.049) which increased by 20% from day 13 to day 17 (paired samples T-test; t = -5.956, p = 0.001).

The pattern of sucrose preference, changed over time (time: $F_{(2,60)}$=26.996, p = 0.001) and was group dependent (group: $F_{(2,60)}$=13.753, p = 0.001). Stressed mice showed significantly less preference for sucrose than controls on day 17 (one way ANOVA, $F_{(1,32)}$=9.837, p = 0.004).

Novelty exploration - circular hole board

The behavioral pattern on the circular hole board differs significantly between the groups (MANOVA: $F_{(12,19)}$=3.521, p = 0.007; Table 3). Stressed mice had a longer latency to the first hole visit and hole dip (one way ANOVA, $F_{(1,31)}$=4.339, p = 0.049; Mann-Whitney, df=32; p = 0.035), while the total number of hole visits did not differ. Interestingly, stressed mice more often used perseveration strategies to visit the holes (one way ANOVA $F_{(1,31)}$=5.269, p = 0.029). Serial strategies, distance walked and velocity were comparable between groups.

Corticosterone response to rat exposure

Corticosterone concentrations before the first (rat) exposure on day 6 were comparable between groups. After rat exposure, higher corticosterone concentrations were

Table 3: Behavioral parameters indicative for exploration of the circular hole board.

Behavioral parameter	Control	Rat stress
Distance (m)	17.5 ± 0.8	17.4 ± 1.4
Speed of moving (cm/s)	5.8 ± 0.3	5.8 ± 0.5
Stops (number)	4.0 ± 0.2	4.5 ± 0.5
Latency first hole visit (s)	9.3 ± 1.0	13.6 ± 1.8*
Latency first hole dip (s)	21.2 ± 3.1	31.6 ± 4.3*
Total hole visits (number)	25.5 ± 1.8	24.4 ± 2.1
% serial	45.8 ± 3.8	57.2 ± 5.0
% perseveration	15.5 ± 3.5	24.4 ± 2.3*
Latency first rim dip (s)	19.5 ± 1.8	25.6 ± 2.8
Rim dips (number)	12.8 ± 1.4	12.0 ± 1.4

Data are expressed as mean ± S.E.M. s: seconds; m: meter

* $p < 0.05$ rat stress vs. controls.

measured as compared to novel cage exposure (baseline = 5.4ng/ml = 100%; t = 60 min: stress: 895.9 ± 68.9 vs. control: 403.9 ± 25.2% increase vs. baseline; Mann-Whitney, df=32; $p = 0.001$).

Body weight

All mice gained weight over the course of the experiment (± 3% increase on day 19; day 1 set to 100% bodyweight). Weight change in direct response to rat exposure or cage placement, was used as a marker for autonomous nervous system activation. Weight loss in stressed mice was significantly stronger (mean ± S.E.M. in mg; control: 43 ± 2 vs. stress: 59 ± 1; one way ANOVA, $F_{(1,31)}=39.894$, $p = 0.001$).

Additional experiment: Sucrose consumption and cage exposure change activity pattern

Mice altered their activity pattern during the dark active period in response to sucrose and exposure to their previous Macrolon Type II housing cage (main effect of group: % time - in open area: $F_{(2,33)}=10.099$, $p = 0.0001$; at feeder $F_{(2,33)}=3.624$, $p = 0.038$) with a different time course over days: interaction day*group (open area $F_{(6,99)}=3.935$, $p = 0.001$; at feeder $F_{(6,99)}=4.331$, $p = 0.001$; data not shown). Sucrose consumption was comparable to the main study (between 10 and 15ml/mouse). Only on day 5, when sucrose was present, time spent near the bottles increased by 50%; time spent at the feeder and in the open area decreased (all $p < 0.05$). Following two cage exposures during the light-on

hours on day 6, the time spent in shelter was increased during the next light-off period. Activity in the open area and at the feeder remained reduced on days 6, 7 and 8. Mice spent less time near the bottle and feeder, while more time was spent inside the shelter (all $p < 0.05$). Taken together, as long as sucrose was available, (foraging) activity was changed but returned to the level of undisturbed control mice thereafter. The removal of the mouse from the home cage PhenoTyper to the "previous housing cage", which we used as control procedure for rat stress in the main experiment, had long-lasting consequences, suppressing the activity pattern of the mice for the next two days.

Discussion

Longitudinal and continuous observation of behavioral activities of mice in their home cage resulted in two main findings: (1) During and after cessation of the rat stress paradigm exploration and foraging patterns changed; consumption of and preference for a sweet solution was less expressed than in control mice. Rat-stressed mice delayed the onset to explore the novel environment of the circular hole board and used perseverative exploration strategies. (2) The control procedure for rat stress, i.e. placing the mice for 1-2 hours in another cage, also resulted in a pronounced decrease and differential pattern of home cage activity. We conclude that experimental manipulations, which are generally considered as "minor", reveal themselves as long-lasting changes in the daily activity pattern of mice. In addition, repeated rat exposure leads to further distinct alterations of behavior in the familiar environment of the home cage, extending even to behavior in novel situations.

Effects of rat exposure on home cage behavior

Repeated, unpredictable and uncontrollable exposure of mice to rats can be considered as chronic psychological stress (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Our results on reduced home cage activity in rat stressed mice are in line with findings from rat studies that reported reduced non-specific activity counts and running wheel activity in relation to stress procedures involving paired housing, food/water deprivation (Gorka et al. 1996), repeated social conflict (Meerlo et al. 1999) and inescapable electric shocks (Desan et al. 1988; Stewart et al. 1990). However, the current set-up allowed specification of the behavioral changes and their development over time.

Stressed mice spent more time in the shelter, spent less time and traveled less distance in the open area of the home cage than mice of the control group. Principal Component Analysis (PCA) revealed that exploration of the open area (Factor 1) is independent from activity related to foraging (Factor 2), i.e. time at the feeder and drinking bottles. Stressed mice showed a decrease in both exploratory and foraging activity. We interpreted these findings in the following way: once a stressed mouse leaves the shelter, its focus is on obtaining food and water, a primary life necessity, while suppressing exploratory activity that is not related to foraging. We conclude that stressed mice kept their actual food and water intake at the same level as control mice, but had adapted a faster collection of the consumables. Food might also have been transported to the shelter. While directly after the rat exposure mice had lost more weight than controls, the body weight remained comparable. This is another indication that stressed mice consumed as much or even more than controls. It might be the case that the food is metabolized differently, resulting in a differential distribution of fat and muscle tissue (Moles et al. 2006).

The effects of stress revealed by the PCA are supported by theories on the economy of behavior. These predict that animal's trade-off their foraging effort in relation to variation of predation risk ("predation risk allocation hypothesis"; (Lima and Bednekoff 1999). Rats are not actual predators of mice, but in nature, mice and rats avoid to share the same living environment. Animals need to forage for food to meet their energy demands, while at the same time they need to minimize the risk of being exposed to a life-threatening situation, i.e. the predator or the threat of a possible predator. We may argue that reduction of exploratory activity has an adaptive value by temporarily decreasing the risk of predation (Norrdahl and Korpimaki 1998).

In contrast to nature, consumables are available *ad lib* in the experimental setting. Moreover, the actual exposure to rats took place in a distinctly different environment. The change in nocturnal activity patterns reveals the long-lasting consequences of the "rat experience" and leaves the impression that also the familiar "safe" environment of the home cage became threatening.

Observing the development of exploration and foraging over the course of rat exposures, we found that the initially lower exploratory activity returned to baseline levels after the tenth rat exposure. However, Factor 2 (foraging activities) remained lower, indicating that the effects of rat stress have distinct effects on the time pattern of behaviors.

Effect of rat stress on sucrose consumption and preference

Measurement of sucrose intake or preference is currently in widespread use in preclinical psychopharmacology for predicting sensitivity to rewards. Animal models of chronic stress in rats generally report a decrease in sucrose consumption as a measure for anhedonia (Dalm et al. 2000; Pothion et al. 2004; Strekalova et al. 2004; Anisman and Matheson 2005; Willner 2005). However, preference for the sweet solution seems to be a more appropriate marker for anhedonia. As expected, mice showed an impressive consumption of (12ml) and preference for (85%) the 5% sucrose solution. Control mice further increased their consumption (up to 144%) and preference over the course of the experiment. Stressed mice decreased the sucrose consumption in the initial phase of rat stress and baseline levels were reached one day after the cessation of rat exposures. Mice housed for 3 weeks with rats did not increase their sucrose consumption either (Calvo-Torrent et al. 1999). Although all mice preferred sucrose over water, the preference for sucrose increased in the course of the experiment only for control but not rat stressed mice. There is a clear time-dependent pattern in the development of increasing consumption of and preference for sucrose in the control mice which is absent in the rat stressed mice. During the stress paradigm (on days 6 and 10) stressed mice spent less time near the bottles than controls, which most likely reduced their fluid intake. However, since the preference of the stressed mice for sucrose did not change in the course of the experiment, and water intake was comparable to controls we feel confident that rat stress affected the hedonic properties of sucrose. Stressed mice did not increase their preference and consumption like controls. Consequently, we assume that the rat stress procedure affected the reward system, also somewhat counteracting the addictive properties of sucrose (Avena et al. 2008).

The volume overload of 300% due to sucrose drinking most likely affected the body's fluid and energy balance. Sucrose by itself is rich in energy, which is utilized directly, stored in adipose tissue or secreted from the body (Peters et al. 2004). Drinking sucrose might have lowered stress-induced corticosterone secretion as shown by Bell and colleagues (Bell et al. 2000). Indeed, absolute corticosterone values in response to rat exposure were lower than measured in previous studies (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Our additional experiment revealed, that the rat stress control procedure (placement in another cage) reduced the nocturnal activity for at least two days, while sucrose overload affected the activity pattern only on the day of consumption. Therefore, the reduced consumption and lesser preference for sucrose is a distinct feature of the rat stressed mice.

Exploration of a novel environment

Rat stressed and control mice showed similar locomotor activity (distance walked and velocity) on the circular hole board, while home cage activity patterns were lower in the stressed mice. Locomotor activity in response to a novel environment cannot be directly compared to activity in a familiar surrounding. In both conditions, a different type of activity is measured, next to the difference in duration and time of measurement. Instead in locomotor activity, the effect of stress was observed at a different level. Interestingly, the exploration pattern of mice on the circular hole board was changed. Stressed mice alternated more often between serial (sequential hole visits) and perseverative (repetitive visits of the same hole) search strategies. While control mice readily set out to explore, stressed mice were slower in starting to visit holes and performing rim dips, indicative for more anxiety-related behavior. Exploration of the holes and the border are important to locate possible routes of escape from the open, unprotected environment. We may assume that the exploration pattern of stressed mice decreases the possibility to locate an escape route. This might also relate to the impairment of cognitive abilities that have been observed in previous studies (Grootendorst et al. 2001b).

Effects on home cage activity of the control mice

The activity of mice *before* the intervention resembled the nocturnal pattern described in previous studies (de Visser et al. 2005; de Visser et al. 2006). Unexpectedly, the control procedure for rat stress, i.e. exposure to a 'familiar' cage for one to two hours reduced activity patterns, albeit to a lesser degree than in the rat stressed group. Others have shown in rats that even routine control procedures like placement into a clean cage, can induce stress system activation and affect behavior (Meerlo et al. 1996; Duke et al. 2001; Balcombe et al. 2004). Placing a rat in a novel cage for 1h in the same room, at the same time when another rat was defeated had a dramatic effect on the body temperature during the day and the activity during the night (Meerlo et al. 1996). Importantly, the results of our additional experiment support the long lasting reduced nocturnal activity of the mice. Exposing the mice to the circular hole board for 5 minutes during the light period of day 19, resulted again in a reduction of activity thereafter. Exploratory and foraging factors returned to the level of day 6, when the mice were exposed for two hours to a novel cage.

Like in our study, most laboratories conduct experiments during the light, inactive period of rodents. Others have shown that the effect of stressors on stress system activation depend on the kind of stress and time of day when applied (Akana et al.

1986; Retana-Marquez et al. 2003). The effects of experimental "control" manipulations and even a short-lasting behavioral test go unnoticed in most studies that compare treatment effects between groups. The longitudinal design of the present study revealed the strong impact of common handling procedures.

Conclusions

The home cage PhenoTyper design includes the measurement of baseline behavior and thus, allowed in a longitudinal setting *within* and *between* group comparisons. We found evidence that already "basic" experimental manipulations like relocation of the mice to another cage, performing a short-lasting behavioral exploration task, have strong and long-lasting influences on the organization of circadian behavioral activity. We now know that the rat stress effect is a combination of rat exposure and accompanying experimental conditions like transport, handling, disturbance of the light phase resting and sleep behavior. Rat exposure resulted in (1) a stronger and differential inhibition of exploration and foraging activity in the home cage than the control procedure, (2) a decreased response to reward expressed by sucrose drinking and sucrose preference and (3) a specific exploration strategy in a novel environment. Behavioral and neuroendocrine changes might still be in the range of adaptive responses to stress. We conclude that our rat exposure design shows the potential for a mouse model of chronic stress and will allow to study and elucidate mechanisms underlying the inhibition of behavior in relation to stress system activation.

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Chapter 7

Post-training reward partially restores chronic stress induced effects in mice

Sergiu Dalm
E. Ronald de Kloet
Melly S. Oitzl

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Abstract

Reduced responsiveness to positive stimuli is a core symptom of depression, known as anhedonia. In the present study, we assessed the expression of anhedonia in our chronic stress mouse model using a subset of read-out parameters. In line with this, we investigated in how far chronic stress would affect the facilitating effect of post-training self-administration of sugar, as we previously observed in naïve mice.

Male C57BL/6J mice were repeatedly and at unpredictable times exposed to rats (no physical contact) over the course of two weeks. Following novelty exploration, (non-) spatial learning and memory processes with and without post-training sugar acting as reinforcer, emotionality, reward sensitivity and corticosterone levels were determined.

We found that (1) the effects of chronic stress persisted beyond the period of the actual rat exposure. (2) Post-training self-administration of sugar as reinforcer improved spatial performance in naïve mice, whereas (3) in stressed mice sugar partially "normalized" the impaired performance to the level of controls without sugar. Chronic stress (4) increased behavioral inhibition in response to novelty; (5) induced dynamic changes in the pattern of circadian corticosterone secretion during the first week after rat stress and (6) increased the intake of sucrose and water. (7) Chronic stress and sugar consumed during spatial training facilitated the memory for the location of the sucrose bottle weeks later.

Concluding, our chronic stress paradigm induces the expression of anhedonia in mice, at different levels of behavior. The behavioral inhibition appears to be long lasting in stressed mice. Interestingly, sugar consumed in close context with spatial learning partially rescued the stress-induced emotional and cognitive impairments. This suggests that reward can ameliorate part of the negative consequences of chronic stress on memory.

Introduction

Chronic stress is considered a vulnerability factor for psychiatric disorders like depression (De Kloet et al. 1998; de Kloet et al. 2005; McEwen 2005). One of the core symptoms of depression is anhedonia, i.e. the reduced reactivity to pleasurable stimuli or positive affect from events or activities that are normally rated as interesting or pleasant (DSM-IV-TR 2000; Holsboer 2000; Bevins and Besheer 2005; Leppanen 2006). Anhedonia is considered to be the result of a disturbance in the detection of and response to positive emotional stimuli. The objective of the current study was to induce a disturbance in emotional processing by exposing mice to a chronic psychological stressor, and to investigate the reactivity to a rewarding stimulus. We measured emotional responsivity, cognitive performance and corticosterone secretion patterns.

Previous studies have shown that repeated exposure of mice to rats, i.e., the 'rat stress' procedure, caused changes in the behavior of mice measured *during* and *directly after* 'rat stress' (Dalm et al. 2009a). The behavioral changes included (i) inhibition of circadian activity patterns in the home cage, (ii) reduced sucrose consumption and inhibition of sucrose preference development and (iii) perseveration of behavior in a novel environment without a change in general locomotor activity. The same 'rat stress' protocol revealed changes in endocrine parameters together with impaired performance in hippocampus-dependent learning tasks (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Recently, we also reported that chronic stress shifted the use of learning strategies towards favoring stimulus response over hippocampus-dependent strategies in mice and man (Schwabe et al. 2008).

To assess whether our chronic stress procedure would induce the expression of anhedonia, we first determined several indicators for anhedonia. For this purpose we exploited the finding that positive stimuli and reward can strengthen memory traces (Huston and Mondadori 1977; Huston and Oitzl 1989; Messier 2004). In line with the theory of reward-effects on memory we have demonstrated that post-training access to sugar facilitated spatial memory of mice in the water maze and the circular hole board task (Dalm et al. 2009b). In the current study we studied the effect of post-training sugar on spatial performance in stressed mice, as indicator for anhedonia.

Another indicator for anhedonia is derived from the consumption of and preference for a sweet solution. We and others have observed inhibition of consumption and preference for a sweet solution in close proximity to stress (Strekalova et al. 2004; Willner 2005; Dalm et al. 2009a). In contrast, long-term effects of stress and elevated glucocorticoids were reported to increase the consumption of and even preference

for sweet solutions (Dallman 2007; Dallman et al. 2007). Others have suggested that exploration patterns in a novel environment may provide leads to reveal the emotional state of the animal (File 2001; Kalueff et al. 2006). Exploration is considered a self-rewarding behavior. While the inhibition of exploration is generally related to anxiety, less exploration might also indicate the loss of hedonic responses, as suggested by Bevins and colleagues (Bevins and Besheer 2005).

We examined the behavior of male C57BL/6J mice over the course of five weeks after cessation of the 'rat stress' procedure. During the first 4 weeks after stress, exploration patterns were determined in the novel environment of the circular hole board, in parallel with the measurement of spatial learning and memory performance, and reversal learning, with and without post-training sugar as reward. At 4 weeks after cessation of the 'rat stress' procedure, we measured the behavioral response to the light-dark box as an indicator for emotion-related behavior. Consumption and preference for a sucrose solution were assessed before, and 5 weeks after 'rat stress'. To substantiate the paradigm of repeated rat exposure as model for chronic stress, we measured circadian corticosterone secretion by taking blood samples three times per day, at one and six days after the last rat exposure.

We hypothesize that (i) chronic stress will impair spatial memory in mice and (ii) the memory facilitating effect of post-training sugar in stressed mice will be absent.

Materials and Methods

Animals

Male C57BL/6J mice (*n* = 40, 10 weeks old) were purchased from Janvier (France). Upon arrival at the animal facilities (Gorlaeus laboratory, LACDR, University of Leiden, The Netherlands), mice were transported to the experimental room to acclimatize for two weeks before the start of the experiment (days 1 - 14). They were housed individually in a temperature (21 ± 1˚C) and humidity (55 ± 5%) controlled room, with food and water *ad libitum*; 12-12h light-dark cycle (lights on at 0700h). Behavioral testing was performed between 0900h and 1400h. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/609/EEC).

Experimental design

Figure 1 depicts the timeline of the experiment. Mice were subjected to two conditions (*n* = 20/group; days 18 - 28); (i) stress: exposure to rats within a 2 week period and (ii) control: remaining undisturbed in the home cage. Endocrine (corticosterone), emotional and cognitive responses were assessed several times throughout the duration of the experiment. The corticosterone concentration was determined three times during the light period: baseline (day 17), and one- and six days after the last rat exposure (days 29 and 34). On day 35, mice were exposed for 5 min to the novel environment of the circular hole board (CHB). The CHB was subsequently used to test acquisition of spatial learning (days 38 – 42) and reversal learning (days 46 – 48). Exploration strategies were assessed on days 35, 45 and 49, i.e., before, after spatial- and after reversal learning. Four weeks after cessation of the stressor (day 56), the behavioral response to the light-dark box environment was assessed. Immediately thereafter a blood sample was withdrawn to determine the novelty-induced corticosterone concentration. A sucrose solution was available for 24 h before (day 15) and after 'rat stress' (day 63). Bodyweight was measured daily from the day of arrival until the end of the experiment.

Day	Experimental manipulation
1 – 11	Single housing in separate control, and stress assigned rooms
12 - 16	Tunnel-training in preparation of CHB training
14	**Sucrose / water** consumption and preference: baseline
17	**Corticosterone** 3 x during light period: baseline
18-28	**Rat stress paradigm**
29	**Corticosterone** 3 x during light period: 1 day post-stress
30 – 33	*Home cage*
34	**Corticosterone** 3 x during light period: 6 days post-stress
35	FET-1 Novelty exposure to the CHB
36 – 37	*Home cage*
38 – 42	**CHB spatial learning and memory**
43 – 44	*Home cage*
45	FET-2
46 – 48	**CHB reversal learning and memory**
49	FET-3
50-55	*Home cage*
56	**Light-dark box** + corticosterone concentration
63-64	**Sucrose / water** consumption and preference

Figure 1
The experimental design of the study. The grey box highlights the time of the chronic stress procedure. Abbreviations: CHB = Circular Hole Board; FET =Free Exploration Trial.

Behavior was recorded on videotape and analyzed using EthoVision Windows 3.1 (Noldus Information and Technology BV, Wageningen, The Netherlands). The image analysis system sampled the position of the mouse 12.5 samples/second. To calculate the distance moved, we set the system to score movement when the mouse moved at least with a velocity of 3.5 cm/second, averaged over 12 samples.

'Rat stress' paradigm

Exposure to a rat profoundly activates the Hypothalamic-Pituitary-Adrenal (HPA) axis of the mouse, resulting in elevated corticosterone concentrations in brain lysate (Linthorst et al. 2000) and blood plasma (Grootendorst et al. 2001b). During the first week (days 18 - 22) of the 'rat stress' paradigm, mice were exposed to rats on 5 consecutive days: one or two hours a day, either morning or afternoon, resulting in a total rat-exposure time of 9h. In the second week (days 26 – 28), two exposures took place: on Tuesday (1h) and Thursday (1h; see also Dalm et al. 2009a).

One rat was placed on top of two mouse cages. Mice and rats were separated by a grid and could see, hear and smell, but not touch each other. Food and water were not available during rat exposure. To reduce predictability of the procedure for the mice, exposures took place at different times during the light phase. Furthermore, the location of the rat and the mouse cages were changed *ad random* within the experimental room. To avoid exposure to the smell of rats, the person who performed the rat stress procedure did not enter the separate housing room of the control mice. Control mice remained in their home cage. To assess the effect of rat exposure on arousal, mice were weighed before and directly after the last rat exposure of the day. Comparable time points were used for weighing the control mice.

Blood sampling and corticosterone measurement

To characterize the effect of the 'rat stress' paradigm at the endocrine level, we used the following procedure: The day before the start of the stress paradigm, and 1 and 6 days after the last rat exposure, a small blood sample was collected from the mice via tail-incision three times during the light period at 0900h, 1300h and 1700h. Briefly, a small incision at the base of the tail with a razor blade allows collection of a < 50 µl blood, within 90 sec after opening of the animal's cage (Dalm et al. 2005). Corticosterone was measured using a commercial ^{125}I-corticosterone radioimmunoassay kit (MP Biomedicals, NY, USA; the intra-assay variability is 7.3%).

Circular hole board

The apparatus is a grey round plate (PVC; diameter = 110cm) with 12 holes (diameter = 5cm) at equal distances from each other and at a distance of 10cm from the rim of the hole to the rim of the plate, situated 1m above the floor. Light intensity on the board surface was 120 lux. All holes could be closed by a lid at a depth of 5cm. During learning trials one hole was open and connected to the home cage of the mouse by an s-shaped-tunnel (diameter = 5cm x 15cm long). Only in close proximity to the hole (head into the hole) the mouse could see if it was open. Turning the board between trials, cleaning the surface before each mouse was placed on the board, and placing the home cage underneath the opposite exit hole during the free exploration trials, served to control odor cues (see for detailed description of the CHB apparatus and procedure (Dalm et al. 2009a)).

Before a trial commenced the board was cleaned with 1% HAc, followed by turning the board clock- or anticlockwise until a randomly determined open hole was at the fixed location of the exit. The location of the exit hole changed between spatial acquisition- and reversal learning. The home cage of the mouse was placed underneath the board and was connected to the exit hole with an s-shaped-tunnel; the home cage was invisible to the mouse on the board. A trial started by placing the mouse in a grey cylinder (PVC, diameter = 10cm; high = 25cm) at the center of the board. After 10s the cylinder was lifted and the mouse could explore the CHB.

Mice were 'pre-trained' three times to climb through the s-shaped-tunnel during the week preceding the 'rat stress' paradigm (days 12 – 16). All mice readily entered and climbed through the tunnel at the third time of 'pre-training'.

Schedule and procedure

Mice were run on the CHB between days 35 – 49. During free exploration trials (FET) all holes were closed by a lid; trials lasted 5 min: FET1: day 35 - novelty exposure; FET2: day 45 – three days after spatial acquisition training; FET3: day 49 – one day after reversal learning. Training trials were divided in (i) spatial acquisition (days 38 – 42): learning the location of an exit hole; (ii) reversal: learning the location of a new exit hole (days 46 – 48). A trial lasted 120s max, and two trials were run per day with an inter-trial-interval of 15 min. If the mouse did not locate the exit hole, it was gently guided towards the exit hole using a grid (20cm × 6cm). A sub-group of control and stressed mice received post-training sugar, upon arrival in their home cage (n = 10/condition).

Overall, mice performed 16 learning trials (10 spatial acquisition and 6 reversals) and 3 FET's. The following parameters were analyzed for the FET's (i) general activity: path length (m), velocity (cm/s), number of holes visited; (ii) search strategies: sequence of hole visits (serial: more than two holes in sequence; perseveration: repeatedly visiting the same hole or alternately visiting two neighbouring holes), latency (s) and path length (m) to the exit holes as learned during spatial acquisition and reversal, number of visits to the exit holes, time spent in the zones (s) comprising of the hole adjacent left and right from the exit hole used during spatial- and reversal learning; (iii) anxiety related: latency (s) to leave the start center, latency (s) to the rim zone, number of rim dips, and number of boli. Training trials were analyzed for: latency (s) to leave the start center, latency (s) and path length (m) to exit hole, velocity (cm/s).

Sugar administration

On the first day of single housing a feeding cup (2.5cm x 2.3cm) was taped to the bottom of the home cage in the corner opposite the nest (Dalm et al. 2008). All mice were familiarized with sugar on days 12 and 16 (i.e., before rat stress and CHB training commenced). The grid of the cage was lifted, the sawdust was removed from the feeding cup, and the sugar (30 mg) was added at 0900h. Mice ate all the sugar within 15 min.

During the second spatial- and reversal training trials of the day, mice had free access to 30 mg sugar. All mice ate the sugar within 15 min after the trial, thus, in close context with the learning trial (Dalm et al. 2009b).

Light-dark box

On day 56 we determined the behavioral response of the mice to placement in the light compartment of the light-dark box and 5 min later blood samples were taken for the measurement of the corticosterone concentration. The plexiglas box was divided into a light- (30cm × 20cm × 25cm; lux = 480) and darker compartment (15cm × 20cm × 25cm; lux = 120). To start, mice were put in a grey cylinder (PVC, diameter = 10cm; height = 25cm), which was always placed in the same corner of the light compartment. After 10s the cylinder was lifted and the mouse was left to explore for 5 min. Thereafter, the box was swept clean with 1% HAc.

As behavioral parameters the time spent (%) and distance moved (cm) in the light compartment were assessed as well as the latency (s) to enter the dark compartment and re-entry into the light compartment.

Sucrose consumption and preference

During sucrose testing mice had access to two bottles in their home cage: containing either water or a 5% sucrose solution. The first measurement of water and sucrose consumption and preference was determined from day 14 to day 15: bottles were weighed before (day 14 at 0900h) and after 24h (day 15 at 0900h). The reduction in weight of the bottles reflected the fluid consumption in ml; the difference in ml drunk from the water vs. the sucrose solution was calculated as percentage and reflects preference. These were taken as baseline values. The second sucrose testing was performed on day 63, which is 45 days after the last rat exposure. After both sucrose testing days, the bottle containing the 5% sucrose solution was replaced by a water bottle. To assess whether sucrose consumption would affect the preference to drink water from a bottle placed at the location of the previously sucrose-containing bottle, water consumption was measured following the second sucrose test, for 24h from day 64 to day 65.

Statistical analysis

Data were subjected to ANOVA (factors: group -controls and stress; treatment: no sugar, sugar, when appropriate with repeated measures followed by a post-hoc LSD test (SPSS 15.0), and presented as mean ± S.E.M. Significance was accepted at $p < 0.05$.

Results

Circular hole board: novelty, exploration and search strategies

One week after 'rat stress', we found a dramatically altered behavioral response of mice exposed to CHB novelty, during (FET-1). Overall, behavior was suppressed in stressed mice, differing significantly between groups ($F_{(14,23)}$=3.60, p = 0.001). General activity as expressed by path length in meters, velocity (cm/sec) and total number of hole visits (Figure 2A-C) was decreased (all $p < 0.01$). Anxiety related behavior (all $p < 0.01$) such as number of rim dips was decreased (Figure 2D) while latency to the rim area was twice as long (stress: 205 ± 25; control: 122 ± 10). Behavior related to search strategies (all $p <$ 0.01) such as time (s) to leave the center (stress: 12.2 ± 1.5; control: 6.5 ± 1.1) and latency to first hole visit (stress: 21.7 ± 4.7; control: 12.5 ± 1.8) were increased in stressed mice. Most remarkably, stressed mice explored the CHB favouring the use of perseveration over serial strategy (%perseveration vs. %serial; stress: 69.1 ± 7.3 vs. 31.3 ± 9.7; control: 52.2 ± 5.9 vs. 40.9 ± 5.4; all $p < 0.01$). An example of the walking pattern of a control and a

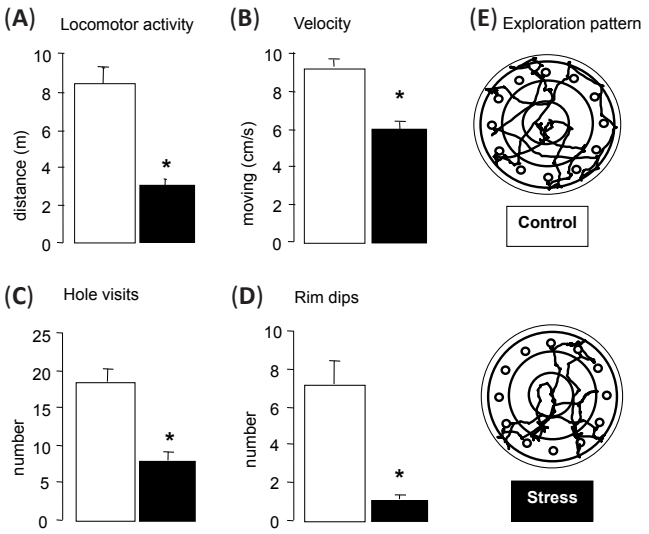

Figure 2
Behavioral responses to the novel environment of the circular hole board were assessed one week after rat stress (5 min free exploration trial - FET-1); (**A**) Locomotor activity expressed as path length in meters; (**B**) velocity (cm/s); (**C**) number of hole visits; (**D**) number of rim dips; (**E**) typical exploration pattern of a control and a stressed mouse. Data represent mean ± S.E.M.; * $p < 0.05$

stressed mouse is given in Figure 2E. Control and stressed mice were randomly assigned to sugar/no sugar subgroups during spatial training on the CHB. These subgroups were comparable in their behavioral response to novelty (data not shown).

Circular hole board: spatial training trials 1 to 10

The learning curve, as expressed by the slope of latency and distance, decreased over trials (latency $F_{(4,72)}$=54.67, p = 0.001; distance $F_{(4,72)}$=6.08, p = 0.001); the pattern was different between control and stressed mice (trials*group: latency $F_{(11,396)}$=3.15, p = 0.001; distance; p = 0.001). Stressed mice displayed a smoother learning curve vs. a see-saw pattern for controls. Walking velocity increased over trials (trials: $F_{(6,216)}$=82.25, p = 0.001). Path length was significantly shorter in stressed mice (trials*group: $F_{(11,396)}$=5.03, p = 0.001; days 1, 2, 3; $p < 0.05$; data not shown). The shorter path length during the first days was paralleled by a slower walking velocity in stressed mice (trials*group: $F_{(6,216)}$=4.41, p = 0.001). On training day 1 and in the first trial of day 2, stressed mice took significantly longer to find the exit hole than controls ($p < 0.05$; Figure 3).

Access to sugar after training resulted in a group-dependent effect on latency to reach the exit hole (Figure 3). Control mice that received sugar showed a smoother learning curve than no-sugar controls. The latter had a typical see-saw pattern, with the first trial of the day longer latencies than the last trial of the previous day. Remarkably, stressed mice showed the opposite: *with* post-training sugar the pattern of performance

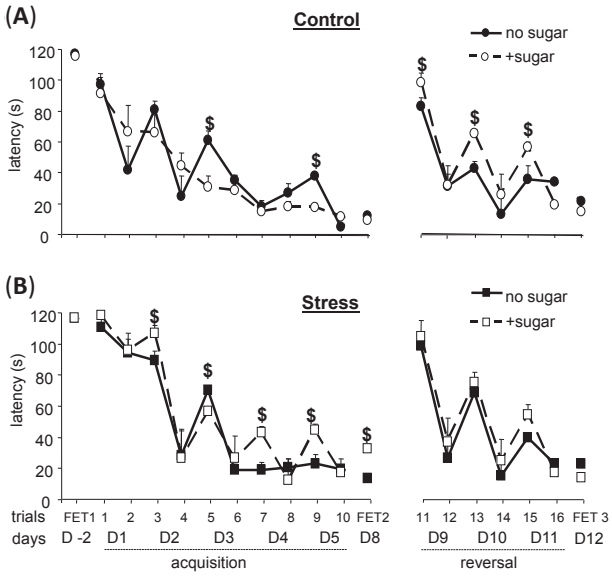

Figure 3

Spatial performance on the CHB (mean of 2 trials/day) expressed as latency to find the exit hole for (A) control mice and (B) stressed mice, during spatial acquisition (training days D1-5) and reversal (training days D9-11). A subgroup of control and stressed mice had free access to sugar (30 mg/day) in their home cage daily after the last training trial. For FET-2 and FET-3, the latency and distance moved relate to the first exit hole visit. Data represent mean ± S.E.M.; * $p < 0.05$ between groups.

was comparable to no-sugar controls; *without* sugar stressed mice showed a smooth learning curve. Post-training sugar did not affect the path length and the walking velocity to the exit hole in either group (trial*group*treatment: $F_{(11,396)}=1.13$, $p > 0.05$).

Over the course of training trials, mice of both groups moved faster away from the start area ($F_{(6,216)}=69.25$, $p = 0.001$; data not shown). However, stressed mice were significantly slower than controls to leave this area not only during FET-1 (before training $p = 0.001$), but also during training days 2, 3 and 5 ($p < 0.05$) and FET-2 (after training; $p = 0.003$). Post-training sugar did not affect the time to leave the start area (time*group*treatment: $F_{(6,216)}=0.56$, $p > 0.05$).

Circular hole board: reversal training trials 11 to 16

During reversal training the exit hole had been relocated from position 3 to 11. The pattern of reversal learning resembles the original learning pattern (Figure 3): long latencies for the first trial, shorter latencies for the second trial of the day. Over days, mice of both groups learned the location of the new exit hole shown by a decrease of latencies over trials ($F_{(3,108)}=37.66$, $p = 0.001$; path length $F_{(3,108)}=9.60$, $p = 0.001$; data not shown). There was no main effect of stress on reversal learning. Control mice showed an effect of post-training sugar: controls with sugar took longer latencies in the first trial of the day ($p <$

0.05). Walking velocity was group dependent (trial*group: $F_{(3,108)}$=3.46, p = 0.019) and significantly lower for stressed mice on days 10 and 11 ($p < 0.05$). Time to leave the start area decreased group-dependently (trials*group: $F_{(3,108)}$=3.70, p = 0.015): stressed mice were significantly slower to leave the start area than controls. Interestingly, post-training sugar had group-dependent effects on this parameter (group*treatment: $F_{(3,108)}$=6.18, p = 0.018). Control mice with sugar were significantly slower to leave the start area than controls in the first trial on days 9, 10 and 11 ($p < 0.05$); also their latencies to the exit hole are longer. Stressed mice with sugar p = 0.041), however, the latencies to the exit hole are the same in both groups.

Behavior during free exploration trials after training

During FET-2 and FET-3 all holes are closed. In comparison to the behavioral response during FET-1 before training, general activity of controls and stressed mice was increased, i.e., path length, speed of moving, and total hole visits. Goal directed behavior became more prominent. The search strategy shifted from perseveration to serial, the latency to the previous learning exit hole decreased, and mice visited the exit hole more often.

Spatial acquisition training differentially affected the behavioral response of control and stressed mice observed in FET-2 (Table 1; MANOVA: $F_{(14, 23)}$=4.54, p = 0.001). Stressed mice were slower than controls to leave the start area and to locate the exit hole. Controls with sugar had less rim dips and visits to the exit hole, yet, were faster in locating the exit tunnel than no-sugar controls. Similarly, stressed mice with sugar had less rim dips than stressed without sugar, while their number of visits to the exit hole was unaffected. The latency to the exit hole of stressed mice with sugar was twice as long as in the stressed no-sugar mice.

The FET-3 following reversal learning revealed group differences in the behavioral response (Table 1: MANOVA; $F_{(14,23)}$=2.11, p = 0.05). Stressed mice made more rim dips than controls, while general activity was similar between groups. Sugar had no effect in the control group. However, stressed mice with sugar had a significantly longer path length, faster walking velocity and more hole visits than stressed mice without sugar (all $p < 0.05$). Furthermore, stressed mice with sugar reached the rim of the board faster and made more rim dips. The search strategy employed was similar between groups. Perseveration was less expressed in stressed mice with sugar than stressed mice without sugar.

Interestingly, memory related parameters differed according to group and treatment. Control mice visited the “new” exit (from the reversal training) about twice as much than the “old” exit (from the initial training); stressed mice visited the “new”

Table 1: Behavioral response during 5min CHB exploration. Assessed three days after spatial training (FET-2) and one day after reversal training (FET-3).

Behavioral parameters	FET–2 after spatial acquisition				FET-3 after reversal training			
	Control		Stress		Control		Stress	
	no-sugar	sugar	no-sugar	sugar	no-sugar	sugar	no-sugar	sugar
General activity								
Path length (m)	15.9 ± 0.6	15.6 ± 0.9	14.8 ± 0.9	**15.0 ± 1.3**$^{\sim}$	13.8 ± 1.1	15.9 ± 0.9	15.4 ± 2.0	18.7 ± 1.1
Speed of moving (cm/s)	13.8 ± 0.3	13.0 ± 0.6	12.8 ± 0.8	**12.1 ± 0.5**$^{\sim}$	13.5 ± 0.3	13.1 ± 0.5	13.6 ± 0.8	14.8 ± 0.4
Total hole visits	54.1± 2.1	53.1 ± 2.9	52.2 ± 5.8	58.2 ± 3.2	48.6 ± 5.1	50.1 ± 1.8	**48.1 ± 4.6**$^{\#}$	63.9 ± 4.0
Search strategy								
Latency (s) from start center	1.5 ± 0.2	**1.7 ± 0.3**$^{\sim}$	**3.0 ± 0.5***	**3.2 ± 0.5***	2.5 ± 0.5	3.4 ± 1.0	3.7 ± 0.6	3.2 ± 0.6
Latency (s) 1ste hole visit	**2.6 ± 0.3**$^{\#}$	4.7 ± 0.6	4.0 ± 0.6	4.4 ± 0.7	**3.0 ± 0.4**$^{\#}$	6.1 ± 0.7	5.0 ± 0.7	3.5 ± 0.3
Latency (s) 1st hole dip	5.0 ± 0.3	**8.0 ± 0.6**$^{\sim}$	**10.8 ± 2.4***	**11.1** ± 1.5*	**6.0 ± 1.0**$^{\#}$	13.7 ± 2.2	10.0 ± 1.6	8.3 ± 1.3
Latency (s) exit hole 3	**12.3 ± 1.3**$^{\sim}$	**9.3 ± 0.9**$^{\sim}$	**13.6 ± 2.4**$^{\#*\sim}$	**32.9 ± 9.0***	27.4 ± 3.4	46.5 ± 13.2	**62.4 ± 17.3**$^{\#}$	20.6 ± 4.2
Number of visits exit hole 3	**8.6 ± 0.7**$^{\sim}$	**6.5 ± 0.5**$^{\sim}$	**9.4 ± 1.4**$^{\sim}$	**8.8 ± 0.7**$^{\sim}$	4.1 ± 0.6	3.5 ± 0.3	**5.1 ± 0.7***	**6.7** ± **0.7***
Latency(s) exit hole 11	n.a.	n.a.	n.a.	n.a.	21.7 ± 7.1	26.8 ± 4.7	23.0 ± 4.6	14.0 ± 2.8
Number of visits exit hole 11	**3.3 ± 0.3**$^{\sim}$	**3.0 ± 0.3**$^{\sim}$	**2.5 ± 0.8**$^{\sim}$	**2.6 ± 0.3**$^{\sim}$	7.1 ± 0.5	5.4 ± 0.6	5.3 ± 0.7	6.9 ± 0.9
% Serial	76.7 ± 3.7	80.0 ± 3.9	75.9 ± 4.9	80.8 ± 3.5	81.0 ± 4.9	88.9 ± 2.6	77.9 ± 4.4	85.7 ± 3.3
% Perseveration	39.8 ± 2.3	36.3 ± 3.8	47.1 ± 4.9	**43.4 ± 4.0**$^{\sim}$	34.2 ± 3.1	35.8 ± 3.1	**38.5 ± 3.9**$^{\#}$	27.7 ± 2.7
Anxiety-related								
Latency (s) to rim	**88.1± 19.7**$^{\sim}$	118.8 ± 17.8	**128.9 ± 7.8***	**165.4 ± 21.8**$^{*\sim}$	**212.3 ± 30.5**$^{\#}$	126.1 ± 18.0	**119.6 ± 11.8**$^{\#*}$	**58.5 ± 12.6***
Number of rim dips	**12.0 ± 2.2**$^{\#\sim}$	7.2 ± 0.9	**8.0 ± 1.4**$^{\#}$	**3.0 ± 0.4**$^{\sim}$	5.3 ± 0.8	6.1 ± 1.0	**11.1 ± 1.3***	**11.9 ± 1.3***

Data are expressed as mean ± S.E.M; $p < 0.05$ * between groups control vs. stress; $^{\#}$ within groups, $^{\sim}$ FET-2 vs. FET-3; n.a. = not applicable
Behavioral parameters that differ significantly are **bold.**

and "old" location comparably often (all $p < 0.05$). It took stressed mice *with* sugar one third of the time to locate the "old" exit hole compared to stressed mice *without* sugar (group*treatment: $F_{(1,36)}=7.37$, $p = 0.023$). Also latencies to "new" and "old" exits were shortest in stressed mice *with* sugar.

Persistence of directed search following spatial training

During 5 days of spatial training mice learned to locate the exit hole. The persistence of search was defined by the percentage time spent in the area at the location of the previously accessible exit hole (15cm radius), during the 5 min of FET-2 (Figure 4). Stress and sugar affected the time spent close to the exit hole. Stressed mice remained longer in the exit area than controls (main effect of group $F_{(1,36)}=5.94$, $p = 0.020$). The effect of sugar on control and stressed mice was opposite (group*treatment $F_{(1,36)}=11.30$, $p = 0.002$): sugar during training increased the time in the exit area in control mice ($p = 0.018$) whilst decreasing it in stressed mice ($p = 0.029$). Consequently, the persistence behavior of control mice *with* sugar was statistically comparable to stressed mice that had received sugar during training.

Behavior in the light-dark box

Four weeks after the last rat exposure, mice were placed in the light compartment of the light-dark box, and tested for light-dark preference. Stressed and control mice responded differently (Table 2: MANOVA: group: $F_{(5,32)}=5.17$, $p = 0.001$). Stressed mice

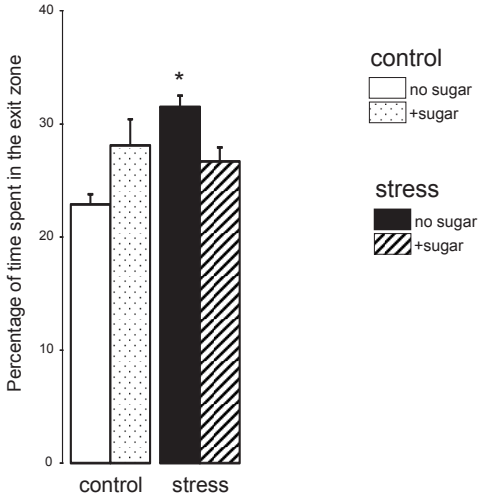

Figure 4

Three days after the last spatial acquisition training trial, the percentage of time spent in the exit zone (15 cm radius) was determined during 5 min of free exploration trial 2 (FET-2). Data represent mean ± S.E.M.; $p < 0.05$ * control vs. stress; ˜ no-sugar vs. sugar.

Table 2: Behavioral parameters expressed in the light area of the light-dark box during 5min exposure.

	Control		Stress	
Behavioral parameters	no-sugar	sugar	no-sugar	sugar
Latency (s) to dark*	7.6 ± 1.3	6.6 ± 0.6	13.1 ± 1.4	**9.7 ± 1.4#**
Latency (s) to light	34.1 ± 4.2	**21.6 ± 1.9#**	29.3 ± 2.4	28.9 ± 3.4
Path length (m)	5.0 ± 0.3	5.0 ± 0.6	6.8 ± 1.3	6.2 ± 0.6
% Time spent *	25.7 ± 1.5	30.1 ± 2.7	42.2 ± 2.2	**32.2 ± 2.8#**
Speed of moving (cm/s)	6.2 ± 0.2	6.2 ± 0.2	5.4 ± 0.3	**6.5 ± 0.3#**

Data represent mean ± S.E.M.; $p < 0.05$ *between groups control vs. stress; # within groups. Behavioral parameters that differ significantly are **bold.**

took more time to enter the dark compartment ($F_{(1,36)}$=12.30, p = 0.001), spent more time in the light compartment ($F_{(1,36)}$=16.58, p = 0.001) and had a longer path length ($F_{(1,36)}$=11.04, p = 0.002) than controls. Walking velocity in the light compartment was comparable between groups.

Sugar had distinct effects on behavior of controls and stressed mice (group*treatment: $F_{(5,32)}$=3.49, p = 0.013). Stressed mice *with* sugar had shorter latencies to the dark compartment and spent less time in the light compartment and their walking velocity was higher than in stressed mice *without* sugar (all $p < 0.01$). Control mice *with* sugar were faster to re-enter, and spent more time in the light compartment than controls *without* sugar (both $p < 0.05$); walking velocity was comparable.

Sucrose consumption and preference

Control and stressed mice preferred sucrose solution over water to a comparable degree. We calculated the difference in fluid intake (5% sucrose-, water- and total fluid consumption in ml) between baseline (day 14; i.e., 4 days before the rat stress paradigm started) and 5 weeks after the last rat exposure (day 63, Table 3). Stressed mice drank more of the sucrose solution and water than controls (group: sucrose $F_{(1,36)}$=9.02, p = 0.005; water $F_{(1,36)}$=4.71, p = 0.037), with a significantly higher total fluid consumption (p = 0.002). Sugar during CHB training had no effect on fluid consumption of controls and stressed mice.

Immediately following the 24h sucrose consumption test on day 63, the sucrose bottle was replaced by a water bottle. Water intake from both bottles was determined 24h later (day 64 - 65). The total water intake was similar, in the range of 10 ml in all groups.

Table 3: Consumption (ml) of and preference (%) for drinking a 5% sucrose solution and water during 24hrs. On day 63 one bottle contained sucrose, the other contained water. On day 64, both bottles contained water.

		Consumption (ml)			**Preference (%)**			
		(day 14 baseline vs. day 63)			day 63		day 64	
group	treatment	sucrose*	water*	total*	sucrose	water	-water-*	-water-
Control	no-sugar	-1.6 ± 1.3	0.6 ± 0.2	-1.0 ± 1.2	88.0 ± 0.6	11.5 ± 0.8	45.9 ± 1.8	54.1 ± 1.8
	sugar	-0.3 ± 1.0	0.8 ± 0.1	0.5 ± 1.0	88.1 ± 0.5	11.9 ± 0.5	50.3 ± 1.7	49.7 ± 1.7
Stress	no-sugar	2.3 ± 0.5	0.9 ± 0.1	1.0 ± 0.1	86.8 ± 0.8	13.2 ± 0.8	52.3 ± 4.7	47.7 ± 2.7
	sugar	1.4 ± 0.6	3.3 ± 0.6	2.5 ± 0.6	87.7 ± 0.6	12.3 ± 0.6	**62.6 ± 2.2**$	37.4 ± 1.2

Data represent mean ± S.E.M.; $p < 0.05$ *between groups controls vs. stress; $ vs. all other groups. Behavioral parameters that differ significantly are **bold.**

However, stressed mice drank more from the water bottle which previously contained sucrose (group: $F_{(1,36)}$=18.92, p = 0.001). Furthermore, stressed mice that had received sugar during CHB training had the highest preference for the water bottle previously containing sucrose (stress *with* sugar vs. all groups $F_{(3,39)}$=10.85, p = 0.002).

Effects of chronic rat stress on corticosterone and body weight

Circadian corticosterone secretion

Rat stress changed the pattern of corticosterone secretion differentially, depending on the post-stress day of measurement (Figure 5A; time*group $F_{(4,114)}$=4.53, p = 0.002). Corticosterone secretion increased over the day (time effect: $F_{(2,114)}$=246.26, p = 0.001). One day post-stress, corticosterone concentrations were higher at 0900h and 1300h compared to the before-stress condition (p = 0.001), but lower at 1700h compared to before-stress and 6-days-post-stress conditions ($p < 0.05$). Remarkably, 6-days post-stress, the overall circadian corticosterone surge during the light period was augmented (Figure 5B: Area_Under_Curve: one-way ANOVA $F_{(2,59)}$=7.52, p = 0.020). In contrast, overall corticosterone concentration during the light period was similar between before-stress and 1-day-post-stress conditions ($p > 0.05$).

Body weight

All mice gained weight over the course of the experiment (about 13%; day 1: controls 24.7 ± 0.2; mice that will be stressed 24.5 ± 0.2; end of experiment controls 27.2 ± 0.2; stressed 28.2 ± 0.3).

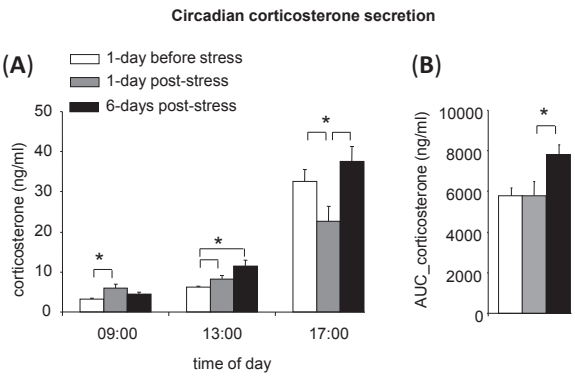

Figure 5

Corticosterone concentrations in blood plasma (ng/ml) determined before and 1 and 6 days after cessation of the stressor during the light period of the day (**A**) at 09:00 a.m., 13:00 and 17:00 p.m.; (**B**) Overall corticosterone concentration during the light period, expressed as Area Under the Curve (AUC_total). Data represent mean ± S.E.M.;* $p < 0.05$ 1-day post-stress vs. before stress and/or 6-days post-stress.

Discussion

The phenotype of chronically stressed mice has a strong resemblance with features of depression in humans. The effects of the chronic 'rat stress' model persisted beyond the period of actual exposure to the rat. One to five weeks after cessation of the stressor, we observed suppression of behavioral reactivity together with altered spatial learning and memory and emotionality. In addition, the pattern of circadian corticosterone secretion showed dynamic changes during the first week after rat stress, culminating in an overall increase in total corticosterone exposure during the light period of day 6. Reward sensitivity was affected as indicated by distinct sensitivity of memory to sugar reward: spatial performance improved in control mice whereas in stressed mice sugar reward "normalized" performance to the level of controls without sugar. Also, an increased sucrose and water intake in stressed mice and preference to drink water at the location of prior sucrose consumption was observed. Remarkably, sugar consumption in close context with spatial learning partially rescued stress-induced emotional and cognitive disturbances, with the effects measured even weeks later in other tasks. Although the increase in sucrose consumption and a similar preference compared to non-stressed mice are not characteristic for anhedonia, they do reflect an alteration in the reward system.

Chronic stress and the expression of anhedonia.

We used a variety of parameters that indicate emotional and cognitive responses in relation to positive stimuli that could be affected by chronic stress: approach behavior, post-training sugar administration and sucrose-preference testing.

Behavioral inhibition

Exploration of novel environments is an essential aspect of behavior. At the same time, the exposure to novelty creates a conflict between approach towards new sources of reward and avoidance of potential treats (Powell et al. 2004; Krebs et al. 2009). Previously, (Dalm et al. 2009a) we exposed chronically stressed mice to the circular hole board two days after the last stressor. Behavioral changes were limited to reduced latency to first hole visit and increased perseveration. In the present study, chronically stressed mice displayed strong behavioral inhibition upon exposure to the novel environment of the circular hole board, one week after cessation of the stressor. The inhibition remained even during recurring training and free exploration trials on the circular hole board, i.e. stressed mice were always slower to leave the start area of the circular hole board. However, over trials the latency to locate the exit hole decreased to the level of non-stressed mice, indicating the learning capability of stressed mice. Interestingly, 5 weeks after the last rat exposure, stressed mice still displayed behavioral inhibition when exposed to the novel environment of the light-dark box. We previously observed a similar response to the light-dark box for stressed mice, even 3 months after cessation of the stressor (Grootendorst et al. 2001b). We may conclude that chronic stress has long-lasting consequences as expressed in different degrees of behavioral inhibition in novel environments.

Approach behavior may yield important information about food and reproduction-possibilities, while an open lit place, for example, is dangerous with regard to predators and has to be avoided (Belzung and Griebel 2001). Indeed, non-stressed mice explored the novel environment of the circular hole board, while also moving away from the brightly lit open space during light-dark box testing. Stressed mice lack the anticipatory responses: their behavior is inhibited and non-adaptive on both the circular hole board and the light-dark box. Chronic stress also reduced the activity of mice in the familiar environment of the home cage (Dalm et al. 2009a). In that study, we showed that the activity was dedicated to foraging (moving to and from the food dispenser) at the expense of moving around in other areas of the cage. It is evident that chronic stress resulted in a shift of approach/avoidance behavior and thus, a lack of

behavioral adaptation in novel environments. Bevins and Besheer (Bevins and Besheer 2005) interpreted such results as changes in reward sensitivity. Therefore, the behavioral inhibition in stressed mice might point towards an alteration in reward that will influence memory formation.

Modulation of learning and memory by post-training reward

Chronic stress and long term exposure to high levels of glucocorticoids are known to alter neuronal morphology and synaptic plasticity in the hippocampus (spatial memory for facts), prefrontal cortex (response selection), striatum (stimulus-response) and amygdala (emotional value of stimuli), amongst other structures, affecting spatial processing (de Kloet et al. 1999; McEwen 1999b; Mizoguchi et al. 2000; Dias-Ferreira et al. 2009; Roozendaal et al. 2009; Conrad 2010). Reward-coding dopaminergic neurons in the hippocampus regulate the motivational drive to explore an environment. They are involved in signaling stimulus novelty and are able to facilitate hippocampus-dependent consolidation memory of novel events (O'Carroll et al. 2006). We had hypothesized that the impact of chronic stress on the modulation of memory by post-training administration of sugar would indicate a change in the reward system of the mice. Post-training reward has been shown to strengthen memory traces (Huston and Mondadori 1977; Huston and Oitzl 1989; Messier 2004). Recently we demonstrated that access to sugar directly post-training resulted in the improved spatial memory of mice in a water maze and circular hole board task (Dalm et al. 2009b).

We will discuss the impact of chronic stress followed by the effects of post-training sugar on learning and memory processes. Chronic stress impaired learning which is in accordance with the literature (Conrad 2010) and our own previous findings on the circular hole board task using an extended training schedule (Grootendorst et al. 2001b). In the present study, two training trials were given each day. The non-stressed controls displayed a see-saw-like pattern of performance, with longer latencies for the first trial of the day compared to the second trial of the previous day (long-term memory). The second trial of the day had short latencies, indicative for intact short-term working memory. Non-stressed mice displayed a smooth learning curve. However, stressed mice had a delay in learning, but did improve their performance from day 3 onwards to the level of non-stressed mice. We regard the extended time in the start area, the slow walking and short distance walked during learning, expressions of behavioral inhibition in stressed mice, as it is also expressed during novelty exposure i.e. the first free exploration trial.

Post-training administration of sugar improved the performance of non-stressed controls. From day 2 onwards, latencies to the exit hole decreased from trial to trial (smooth learning curve), while controls without sugar were slower during the first trials of the training trials, resulting in a kind of "seesaw" pattern of performance. Treating the stressed mice with sugar revealed an interesting "normalization" of behavior. These mice displayed the same see-saw pattern of performance as non-stressed controls without sugar. However, this was a partial similarity to the behavior of controls as stressed mice with sugar had longer latencies during all first training trials of the day, and non-stressed controls improved over days. Nonetheless, post-training access to sugar could alleviate the effects of chronic stress and partially "normalize" the performance to the level of non-stressed mice. We consider this effect to be additional support for a chronic stress-induced alteration of the reward system. Concluding, the rewarding effects of sugar on memory depend on the prior life history, having experienced chronic stress or not.

In addition to a series of training trials over days, we challenged the mice with two conditions that require behavioral flexibility, changing behavior and learning strategies: (1) the exit hole is not available any more during the free exploration trials after spatial acquisition training; (2) the location of the exit hole was changed, i.e., reversal trials. The free exploration trials revealed that stressed mice use a more perservative strategy and are less flexible (returned more often to the same hole, remained longer in the area of the exit hole), as opposed to the more efficient serial strategy employed by the non-stressed mice. Focusing on the aspect of learning strategies, we recently reported that our chronic stress paradigm produces a shift in the use of search strategies by favoring stimulus-response over spatial learning strategies in mice and man (Schwabe et al. 2008). Others (Dias-Ferreira et al. 2009) demonstrated in rats that chronic social stress caused a reorganization of the frontostriatal neuronal network and led to a bias of behavioral strategies towards habit (i.e., stimulus-response) learning. Acquiring the novel location of the exit hole is achieved by all mice. The free exploration trial following reversal training revealed that stressed mice returned to the original exit hole just as often as they returned to the new one, while non-stressed mice favored the new exit location. We might conclude that reversal learning is superior in the non-stressed mice. Surprisingly, latencies to exit were prolonged in non-stressed mice with sugar during reversal learning. Speculating, it might be that the original memory trace of the non-stressed mice with sugar is stronger than in the non-stressed without sugar, and therefore, interferes with the acquisition of new memory. For the stressed mice, post-training sugar has no apparent effect on reversal learning expressed by latencies to the new exit hole. The free exploration trial revealed behaviors of stressed mice with sugar

that indicate increased flexibility, such as less perseveration and early approach of the rim area.

Emotions affect memory. It might be argued that changes in emotions, such as increased anxiety, contribute to the altered performance of the stressed mice. Behaviors related to anxiety and reduced risk-taking e.g., reduced speed of movements, reduced exploration, and not visiting the rim area of the circular hole board, would support such a notion. In contrast, elevated anxiety is not expressed by stressed mice which remain long in the lit area of the light-dark box. Therefore, we prefer to consider a change in the behavioral inhibition, the balance between approach and avoidance as an acceptable operationalisation of behavior.

Sucrose consumption and preference

The most common procedure to determine whether anhedonia has been induced in animals is the measurement of sucrose consumption and/or preference. Chronic stress most often decreases sucrose consumption when tested during, and in close context with the applied stressor (Pothion et al. 2004; Anisman and Matheson 2005). In our previous study, chronic stress reduced sucrose consumption during the stress period and delayed the development of sucrose preference measured one day after the last stressor (Dalm et al. 2009a). We can interpret this result as stress-induced anhedonia. In the present study we measured sucrose consumption 35 days after cessation of the stressor. Stressed mice consumed more volume of both sucrose and water. In contrast with our previous study, the sucrose consumption was not an indicator for anhedonia. Stressed mice even drink more fluid than non-stressed mice with the same preference for sucrose (88%) over water. In fact, we find a stress-induced increase of caloric intake. It is known that glucocorticoids stimulate behaviors that are mediated by the dopaminergic mesolimbic “reward” pathways, and increase the intake of food with high carbohydrate and fat (Dallman et al. 2007), so-called “comfort” food, which contributes to the development of obesity.

Remarkably and at this time unexplainable is the finding that stressed mice that had received sugar during spatial training weeks before, preferred to drink water at the location where they had drunk sucrose the day before. Did they perceive the taste of sugar as highly rewarding, strengthening the memory for this location? It would be of great interest to study the time-dependent effects of chronic stress with respect to stress-induced metabolic changes and food intake.

Conclusion

Chronic stress has immediate and long lasting consequences for behavior, emotional and cognitive abilities. Especially the behavioral inhibition seems to become part of the daily repertoire of responses elicited by novelty, as well as in the familiar environment of the home cage. Corticosterone secretion patterns change, manifested as higher corticosterone levels during the day, within a week after cessation of the chronic stress procedure. Post-training reward in close context with a spatial learning task could partially rescue the chronic stress-induced behavioral changes that reflect emotions and cognitive processes.

We conclude that our chronic stress model results in behavioral and neuroendocrine features that might contribute to the development of stress-related psychopathologies, such as depression and anxiety disorders. Introducing context-related periods of reward, as we did in relation to spatial memory formation, can ameliorate some of the chronic stress effects. Several parameters of behavior became comparable between stressed and non-stressed control mice. Other features, such as the stress-induced increased consumption of sucrose and water were not counteracted. Sugar as a reward even strengthened the memory for the location of the sucrose. This could indicate a possibility for craving and thereby affecting consumption of high caloric nutrients in the future. Our study has provided some insight into the complex interaction of reward and stress. While there are clear positive consequences on memory formation, metabolic effects in relation to chronic stress need more attention in future studies.

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Chapter 8

Chronic stress modulates the use of spatial and stimulus-response learning strategies in mice and man

Lars Schwabe
Sergiu Dalm
Hartmut Schächinger
Melly S. Oitzl

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Abstract

Acute stress modulates multiple memory systems in favor of caudate nucleus-dependent stimulus-response and at the expense of hippocampus-dependent spatial learning and memory. We examined in mice and humans whether chronic stress has similar consequences.

Male C57BL/6J mice that had been repeatedly exposed to rats ('rat stress') used in the circular hole board task significantly more often a stimulus-response strategy (33%) than control mice (0%). While velocity was increased, differences in latency to exit hole, distance moved or number of holes visited were not observed. Increased velocity and performance during retention trials one day later indicates altered emotionality and motivation to explore in rat stressed mice. Forty healthy young men and women were split into "high chronic stress" and "low chronic stress" groups based on their answers in a chronic stress questionnaire ("Trier Inventory of Chronic Stress"-TICS) and trained in a 2D task. A test trial immediately after training revealed that participants of the "high chronic stress" group used the S-R strategy significantly more often (94%) than participants of the "low chronic stress" group (52%). Verbal self-reports confirmed the strategy derived from participants' choice in the test-trial.

Learning performance was unaffected by the chronic stress level. We conclude that one consequence of chronic stress is the shift to more rigid stimulus-response learning, that is accompanied by changes in motivational factors in mice.

Introduction

Memory consists of multiple systems which differ regarding the processed kind of information, the performed operations and the underlying neural structure (Gabrieli 1998; Squire 2004a). "Cognitive" memory supports the acquisition of flexible, consciously accessible knowledge, such as the memory of your last birthday party, and is based on the medial temporal lobe, in particular the hippocampus (Scoville and Milner 1957; Eichenbaum 2004). "Habit" memory, on the other hand, processes simple stimulus-response (S-R) associations, such as "stop your car when the traffic lights are red". It is not necessarily accessible and relies on the caudate nucleus (Knowlton et al. 1996; Jog et al. 1999).

Hippocampus- and caudate-based systems work in parallel and process information simultaneously (Mizumori et al. 2004). The nature of interactions between these systems has been described as cooperative by some authors (Voermans et al. 2004) and competitive by others (Poldrack and Packard 2003) raising the question which factors coordinate their use. Recent findings suggested that stress plays a critical role in the modulation of multiple memory systems. Acute stress prior to training in a task that could be acquired by a hippocampus-based spatial and a caudate-based S-R strategy favored caudate-based learning both in rodents and humans (Kim et al. 2001; Packard and Wingard 2004; Schwabe et al. 2007). This stress-induced modulation of hippocampus-dependent and caudate-dependent systems is assumed to be mediated by the amygdala (Packard & Wingard, 2004). Effects of prolonged or repeated periods of stress on the modulation of caudate-dependent and hippocampus-dependent learning have not been studied yet. This however, would be particularly valuable since chronic stress has been related to psychiatric disorders such as depression (for a review: Willner 1997).

Chronic stress impairs hippocampus-dependent learning and memory (Bodnoff et al. 1995; Kleen et al. 2006). Non-hippocampal memory systems respond differently. Working memory was not affected after repeated restraint stress (Kleen et al. 2006), but fear memory was even strengthened following a prolonged stress period (Conrad et al. 1999). Interestingly, Wright and Conrad (Wright and Conrad 2005a) demonstrated in chronically stressed rats that salient intramaze cues prevented impaired performance in a spatial Y-maze task. We suggest that the introduction of intramaze cues allowed for S-R learning and thus, compensated for impairment of spatial functions. Consequently, we hypothesize that chronic stress modulates multiple memory systems in favor of caudate-based and at the expense of hippocampus-based learning.

To test this hypothesis, we used experimental designs that provide a single proximal and multiple distal cues for learning the task, i.e. allowing stimulus-response learning and spatial learning. Changing the position of the proximal cue in the last trial of the learning session revealed the used strategy in mice and humans. First, we examined in mice the effect of chronic stress (i.e. by repeatedly exposing the mouse to a rat, but separated by a partition) on the use of spatial and S-R learning strategies during the acquisition of a circular hole board task, followed by a retention test 24hrs later. Second, we examined in humans the influence of self reported chronic stress as assessed by the Trier Inventory of Chronic Stress (TICS) on the learning strategy used in a 2D spatial task in which the position of a win-field could be acquired by spatial and S-R strategies.

Materials and Methods

Mouse study

Animals

Male C57BL/6J mice (n = 24, 12 weeks old; purchased from Charles River, The Netherlands) were single-housed in a temperature- (21 ± 1°C) and humidity-controlled room on a 12-12h light-dark cycle (lights on at 0700h) with *ad libitum* access to food and water. Behavioral experiments were performed in the same room. Three times during the week before training started, mice were 'pretrained' to climb through an S-shaped tube into their home cage after weighing. Experiments were approved by the Local Committee for Animal Health, Ethics and Research of the University of Leiden. Animal care was conducted in accordance with the EC Council Directive of 24 November 1986 (86/009/EEC).

Experimental design

Five days prior to the beginning of the rat stress, general activity and exploratory behavior of mice were assessed on the circular hole board. Animals were randomly assigned to one of two conditions: control (n = 12) and 'rat stress' (n = 12; see below). Mice of the "rat stress" group were repeatedly exposed to a rat for 1 to 2hrs a day during 2 weeks. Seven days after the last rat exposure mice started with the circular hole board (CHB) task. Twenty-four hours after training retention performance was tested. Testing took place between 0800 and 1230h. One day later, mice were sacrificed between 0800 and 1000h. The experimenter was unaware of the previous treatment of the animals. Behavior

was recorded on videotape and analyzed by EthoVision 1.95 (Noldus Information and Technology BV, Wageningen, The Netherlands). This image analysis system sampled the position of an animal 12.5 times per second; to calculate the distance moved we chose for a minimal distance between samples of 3cm.

Rat stress paradigm

In nature, mice and rats avoid each other. Exposure to a rat is highly stressful for a mouse (Linthorst et al. 2000). In the first week, mice were exposed to male Wistar rats on 5 consecutive days (1-2h per day resulting in 9hrs in the first week). In the second week, mice were confronted with rats on Tuesday and Thursday for 1h. This time schedule was chosen to increase unpredictability and uncontrollability which are key stress components (Dickerson and Kemeny 2004). Rats were placed in a cage with a grid floor and Plexiglas walls on the top of two mouse cages which were covered by a grid. Thus, mice and rats could hear, see and smell, but not touch each other. During exposure to rats mice were kept in another cage than their home cage (but always the same cage for confrontation with rats) without food and water. The rat stress took place during the light phase (0700 to 1900h) in a room adjacent to the housing room. Previous studies using the same stress protocol showed that it induces reliable features of chronic stress expressed e.g., by reduced body weight, changes in corticosterone secretion and alteration in hippocampal corticosteroid receptor expression, strain-dependent alterations in learning and memory and motivation to explore (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Mice of the control group (naïve) were housed in their home cage.

Learning task

Apparatus: The circular hole board (CHB) is a revolvable white Plexiglas plate (diameter: 110cm) with twelve holes (diameter: 5cm) at equal distance to each other, 10cm from the rim. It is situated 1m above the floor (see Figure 1A; light intensity at the level of the platform 120lux). Holes can be closed by a lid at a depth of 5cm. Whether a hole is open or not can be recognized by the mouse if it puts its head over the edge of the hole. If open, the hole provides access to the home cage of the mouse via an s-shaped 15cm long tunnel (diameter: 5cm). Since mice avoid open, illuminated areas, it is reasonable to assume that mice are motivated to leave the platform. Same as in landmark studies in the field (De Quervain et al. 1998; Winocur et al. 2005) numerous distal cues in the room allowed spatial orientation.

(**A**)

(**B**)

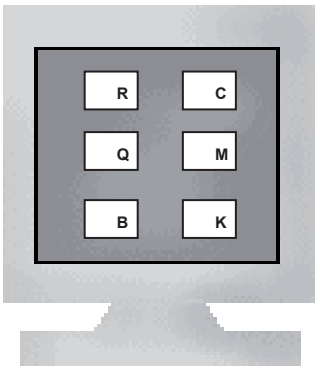

Figure 1
Apparatus used in the mouse **(A)** and human study **(B)**. Mice were trained to find an exit hole. They could use either a spatial (room cues) or a stimulus-response strategy (bottle). Relocation of the bottle in the test trial revealed the used strategy. In the human study, participants could identify the position a "win-field" with a spatial (right column, second row) or a stimulus-response (stimulus: letter M) strategy. Changing the arrangement of the letters in the test trial allowed revealed the employed strategy.

Procedure: At the beginning of each trial mice were placed in a cylinder (Plexiglas; 25cm high, 10cm in diameter) located at the centre of the CHB. After 5s the cylinder was lifted and mice could explore the board and exit through the open hole. There was just one open hole during training which was at the same location in all six training trials, next to a bottle (transparent 0.5 liter bottle filled with water; 22cm high, 5cm in diameter; placed at the rim of the board, see Figure 1A). Thus, the exit hole could be located via two strategies: mice could use cues in the room (spatial strategy) or they could use the bottle as a proximal cue (S-R strategy). If a mouse did not enter the exit hole within 120s the experimenter guided it there by a grid (20cm x 6cm). Six training trials were given (inter-trial interval: 15 min). This relatively low number of trials was chosen to avoid training to asymptotic performance which would promote the use of an S-R strategy (Packard &

McGaugh, 1996). Fifteen minutes after the last training trial a test trial (trial 7) revealed the strategy. In this test trial, the bottle was relocated next to the hole opposite to the position of the exit hole during training. Now, two exit holes were available: one next to the novel position of the bottle and one at the position of the exit hole during training. Leaving the CHB via the hole next to the bottle was classified as S-R strategy. Leaving the board through the hole in the old position was classified as spatial strategy. To avoid that behavior during the test trial could be biased by odor cues; the bedding of the home cage of one mouse was distributed over two cages each placed under one hole.

On the following day, three retention test trials were given which were exactly the same as the test trial. After each mouse, the board was wiped with 1% HAc solution to spread odor cues and turned clockwise until another hole was at the location of the exit.

Five days prior to the beginning of the rat stress, general activity and exploratory behavior of mice were assessed. All holes were closed (the bottle was at the location where it will be during training). After 5 min the hole next to the bottle was opened and the mouse was gently guided by a grid (20cm x 6cm) towards the exit hole. Mice did not show a bias for a certain location on the board during the exploration.

Thymus, Adrenals and Plasma Corticosterone

At the end of the experiment, mice were decapitated under basal resting conditions; thymus and adrenals were removed and weighed to verify the success of the stress protocol. Adrenal weights of three and thymus weights of two animals are missing. Furthermore, blood obtained via decapitation was collected individually in capillaries (coated with potassium-EDTA, Sarstedt, Germany) and stored frozen at -20°C. Plasma corticosterone concentrations were determined (in 10µl plasma) using commercially available radioimmunoassay kits with ^{125}I-corticosterone (MP Biomedicals Inc. Europe, Belgium; sensitivity 3ng/ml; intra-assay variability 7%).

Human study

Participants

Forty young healthy students (21 females, 19 males) aged between 20 and 32 years (mean: 23.9 yrs; SD = 2.7 yrs) participated in this study. Participants were recruited at the University of Trier and got paid a moderate monetary compensation. Exclusion criteria were checked in an initial interview and comprised current or chronic mental or substance use disorders, current physical disease as well as the use of medication

that affects central nervous and endocrine systems. All participants provided written informed consent.

Trier Inventory of Chronic Stress (TICS)

The Trier Inventory of Chronic Stress (TICS; Schulz and Schlotz 1999; Schulz et al. 2004) is a valid and reliable German 57-item questionnaire that was designed to measure 9 aspects of chronic stress: "work overload", "social overload", "pressure to succeed", "work discontent", "excessive work demand", "lack of social recognition", "social stresses", "social isolation" and "chronic concern". Items are descriptions of experiences such as "I have to finish too many things" and people are asked to specify on a 5-point rating scale ("never", "infrequent", "sometimes", "frequent", very frequent") how often they made the referring experience within the last 3 months. The time required to complete the TICS is 10 to 15 min.

"*High vs. low chronic stress":* To assess the effect of chronic stress, we calculated a chronic stress score by adding up the scores of the nine TICS scales. Next, we performed a median-split and assigned the participants with a chronic stress score higher than the median to the "high chronic stress" group and the participants with a chronic stress score lower than the median to the "low chronic stress" group. It is important to note that we tested healthy subjects and that the measured chronic stress scores were in a normal, non-pathological range. Our labels "low chronic stress" vs. "high chronic stress" refer to the median in the present study. They do not indicate low vs. high chronic stress in an absolute sense.

Learning task

Participants were presented six rectangles (6cm x 4cm) arranged in two columns on a customary 17" computer screen (Figure 1B). Each of the rectangles was marked by one letter: R,C,Q,M,B,K. Participants were told that one of these rectangles is a win-field and asked to click with the mouse cursor at the rectangle which they thought would be the win-field. Immediately thereafter, either a "win" or "blank" window popped up, serving as positive or negative feedback. Per trial one rectangle could be chosen. At the end of the experiment, participants received 50 Euro-Cent for each trial in which the win-field was found. The arrangement of the letters was the same in all 14 training trials. Participants were not informed that the win-field was always in the same position (marked by the letter M, right column middle). Thus, there were two possible strategies to identify the win-field: participants could learn the position of the win-field via the

association with the letter (S-R strategy) or they could use a spatial strategy, i.e. they could use the spatial location (right column, middle). Fourteen training trials were given (inter-trial interval: about 30s). Previous findings showed that the used learning strategy is a function of practice with participants using spatial learning at the beginning of a task and S-R learning after extensive practice (Iaria et al. 2003). We chose the number of training trials to assess participants' performance rather early in this process. Participants were classified as "learners" when they chose the correct field three times in a row and did not switch to another field in a subsequent trial. Trial 15 was the test trial - here, the six letters were rearranged. Choosing the field with the letter M in the novel position was classified as S-R strategy. Choosing the field in the position where the win-field had been during all training trials (second column, middle) was classified as place strategy. Trials 1 to 15 were performed within 8 to 10 min.

The experimental procedure was created with the help of the software E-prime (Psychological Software Tools, Inc.; Pittsburgh, USA). Behavioral analyses focused on reaction times and the chosen field in the test trial.

Verbal report

Subsequent to participants' choice in the test trial but before they received feedback, participants were asked (i) to indicate on a scale from 0 to 100 how certain they feel that the chosen field is the win-field (0 - "absolutely uncertain"; 100 - "absolutely certain") and (ii) to explain why they have decided for the chosen field.

Statistical analysis

Data were subjected to χ^2-test, mixed-design ANOVA or t-test, as appropriate. Reported p-values are two-tailed and $p < 0.05$ was accepted as significance. All calculations were done with the statistics software SPSS (version 14.0; SPSS Inc.).

Results

Chronic stress favors the use of stimulus-response learning strategies in mice

Learning strategy: Mice were repeatedly exposed to a rat over a period of 2 weeks, a procedure with long-lasting and profound effects on the stress responsive system and behavior of mice (Grootendorst et al. 2001a; Grootendorst et al. 2001b). One week after the last contact with a rat, mice were trained in six trials on a circular hole board (CHB)

to find an open hole providing access to the home cage. This hole was marked by a cue (a bottle) and could thus be located by caudate-dependent S-R *and* hippocampus-dependent spatial strategies (Figure 1A). Relocation of the cue to another hole in trial 7 (test trial) revealed the applied strategy. Control mice were housed in their homecage until behavioral testing started. They had been never exposed to rats. Groups differed significantly regarding the used learning strategy in the test trial ($\chi^2(1)=4.80$, $p < 0.03$; Figure 2). One third of the chronically stressed mice used an S-R strategy, while – in line with the findings of Kim and colleagues (2001) - all naïve control mice applied the spatial strategy.

Performance: Decreasing latencies and number of holes visited over trials indicated learning performance in both groups (latency: $F_{(5,110)}=8.37$, $p < 0.001$; number of holes visited: $F_{(5,110)}=4.04$, $p < 0.01$; Figure 3). The learning curve of the mice shows that no asymptote is reached which would be indicative for "extensive training". As shown in Figure 3, mice made on average 2-3 errors before selecting the correct hole in the last training trials. Nevertheless, search was not at all random as suggested by the fact that then proportion of time in which mice were in the correct quadrant of the CHB increased significantly over trials ($F_{(5,110)}=2.32$, $p < 0.05$). There were no group

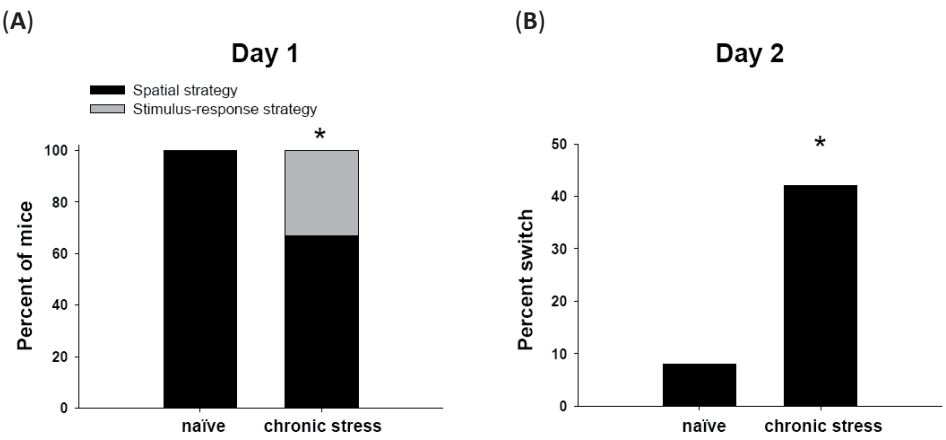

Figure 2

(A) Percent of chronically stressed and naive mice that used a spatial or stimulus-response strategy in the test trial on day 1. Chronic stress changed the used strategy towards more stimulus-response learning. **(B)** Percent of mice that chose a different hole in the first trial on day 2 than in the test trial on day 1. Behavior of chronically stressed mice was less predictable than that of controls. * $p \leq 0.05$.

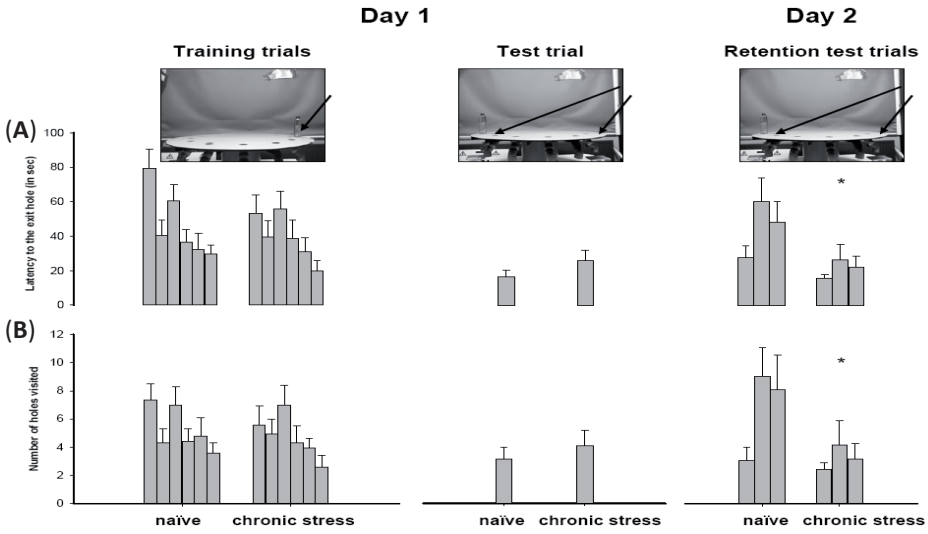

Figure 3

Mice: Latencies to the exit hole (A) and number of holes (B) visited during the six training trials and the test trial on day 1, and during the three retention trials on day 2. Chronic stress affected neither the latencies nor the number of holes visited on day 1 but reduced both parameters on day 2. Inset: circular hole board with the location of the bottle, arrows point at the exit hole(s). Data represent Mean ± S.E.M. * $p < 0.05$.

differences in the latency to the exit hole, neither during training ($F_{(1,22)}$=0.55, p = 0.47; group × trial: $F_{(5,110)}$=0.29, p = 0.91) nor in the test trial (t(22)=0.77, p = 0.57). Similarly, there was no effect of chronic stress on the number of holes visited during training ($F_{(1,22)}$=0.40, p = 0.53; group × trial: $F_{(5,110)}$=0.33, p = 0.89) or in the test trial (t(22)=0.66, p = 0.52). However, chronically stressed mice moved significantly faster during training than controls (velocity: $F_{(1,22)}$=5.37, p = 0.03). This pattern did not change when spatial learners of the chronic stress and control group were compared (all $F < 1.5$, all $p > 0.25$; except velocity: $F_{(1,17)}$=4.79, $p < 0.05$)

Interestingly, relocation of the cue in the test trial caused a decrease in latency in controls but an increase in chronically stressed mice underlining the rigidity and reduced flexibility of the behavior of chronically stressed mice (trial (t6, test trial) × group: $F_{(1,22)}$=4.58, $p < 0.04$; Table 1). A similar pattern was observed for velocities: while chronically stressed mice had decreasing velocities from trial 6 to the test trial, naïve mice increased velocity from trial 6 to the test trial (trial (t6, test trial) × group: $F_{(1,22)}$=5.49, p = 0.03; Table 1). Chronically stressed mice visited more holes after cue relocation in the

Table 1: Velocities and latencies to exit hole of naïve control and chronically stressed mice in the last training trial and the test trial. Controls had decreasing latencies and increasing velocity in response to cue relocation in the test trial; chronically stressed mice showed the opposite pattern (chronic stress × trial: velocity - $F_{(1,22)}$=4.58, p < 0.04; latency - $F_{(1,22)}$=5.49, p = 0.03; holes visited - $F_{(1,22)}$=1.11, n.s.). * Significantly lower than in the test trial (p < 0.05).

	Naïve		Chronic stress	
	Last training trial	Test trial	Last training trial	Test trial
Velocity (in cm/sec)	8.2 ± 0.5*	9.8 ± 0.6	9.8 ± 0.9	8.5 ± 0.6
Latency (in sec)	26.0 ± 5.8	16.4 ± 3.7	20.0 ± 5.3	29.6 ± 5.4
Holes visited	3.6 ± 0.7	3.1 ± 0.8	2.5 ± 0.8	4.1 ± 1.0

test trial than in the last training trial, whereas naïve mice tended to visit fewer holes in the test trial than in trial 6. However, the referring interaction effect failed to reach statistical significance (trial (t6, test trial) × group: $F_{(1,22)}$=1.11, p = 0.26; Table 1).

Retention performance: Twenty-four hours later, mice performed three trials. Two exits were available: one at the bottle (same as during test trial 7), the other at the position of the training trials 1-6. Both groups used mainly the hole at the position of the training trials to access their home cage. However, chronically stressed mice switched their strategy significantly more often from the test trial to the first trial on day 2 (42% chronically stressed vs. 8% naive mice: $\chi^2(1)$=3.56, p = 0.05; Figure 2B). A mixed-design ANOVA for the latencies to the exit hole revealed a significant group and trial effect. Both groups showed shorter latencies in the first than in the following trials ($F_{(2,44)}$=3.30, p = 0.05). Chronically stressed mice had shorter latencies than controls, especially in trials 2 and 3 ($F_{(1,22)}$=7.86, p = 0.01; Figure 3). The same pattern was found for distance moved and the number of holes visited (all p-values < 0.03). There was no trial effect on the animals' velocity ($F_{(2,44)}$=0.37, p = 0.69); like 24hrs before, chronically stressed mice moved significantly faster than controls ($F_{(1,22)}$=8.57, p < 0.01). When only spatial learners of the chronic stress group were considered, group differences remained unchanged (Fs > 5, p's < 0.05).

To assess basal exploratory behavior and locomotion, all mice had spent 5 min on the CHB (all holes closed), one week before the rat stress started. No group differences regarding the number of holes visited and the latency to the hole which provided access to the home cage in the training trials three weeks later were observed (both t-values < 1.04, and p's > 0.30).

Learning strategy and performance within the stressed group: Mice were classified as spatial and SR learners based on their performance in the test trial. Spatial

and S-R learners had similar latencies in trials 1 to 6 and in the test trial ($F_{(1,9)}$=0.02, p = 0.97). Over the three trials on day 2, S-R learners decreased their latencies to the exit hole, the distances walked and the numbers of holes visited, whereas these parameters increased in the spatial learners. Thus, spatial learners of the stress group showed the same performance pattern as spatial learners of the control group.

*Endocrine parameters (*Figure 4*):* More than one week after the last rat exposure, rat stressed mice had significantly enlarged adrenals (t(19)=2.31, p = 0.03); thymus weight was lower but did not differ significantly between rat stressed and control groups (M ± S.E.M. in mg; controls: 42.83 ± 2.22, chronic stress: 38.93 ± 2.67; t(21)=1.11, p = 0.27). Basal plasma corticosterone under resting conditions was significantly increased in the rat stressed group (t(22)=3.80, p = 0.001). The three parameters indicate the success of the chronic stress protocol.

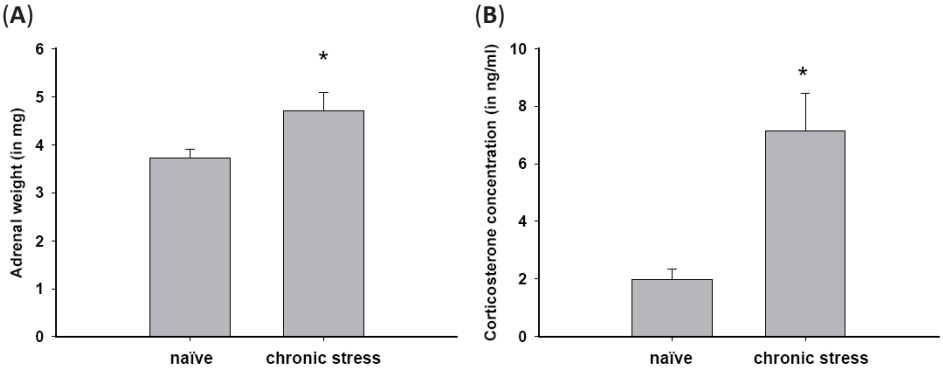

Figure 4

Chronic stress caused a significant increase in **(A)** adrenal weight and **(B)** plasma corticosterone suggesting that the use rat stress protocol was effective. * p < 0.05.

Chronic stress favors the use of stimulus-response learning strategies in humans

Chronic stress and learning strategy: Forty young healthy humans were given a questionnaire (Trier Inventory of Chronic Stress, TICS) measuring chronic stress and trained in a 2D spatial task. They had to locate the one win-field (marked by a cue) out of six (Figure 1B) in 14 trials using spatial or stimulus-based learning strategies. Relocation of the cue in the test trial (trial 15) revealed the applied strategy. Twenty-six participants (65 percent) used an S-R strategy, 9 (23 percent) employed a spatial strategy, 5 (12 percent) chose neither the S-R nor the spatial option ("non-learners").

Participants had been assigned to high vs. low chronic stress groups (n = 20 per group; "low chronic stress" – median: 435, range: 346 - 461; "high chronic stress" – median: 489, range: 463 - 579; Figure 5A). The number of non-learners did not differ between groups: two vs. three in the high vs. low chronic stress group. Importantly, "high chronic stress" changed the used learning strategy significantly ($\chi^2(1)=5.02$, p = 0.025; Figure 5B). Ninety-four percent (17 out of 18) of the learners in the "high chronic stress" group applied an S-R strategy in the test trial while the S-R strategy was used by 52 percent (9 out of 17) of the learners in the "low chronic stress" group.

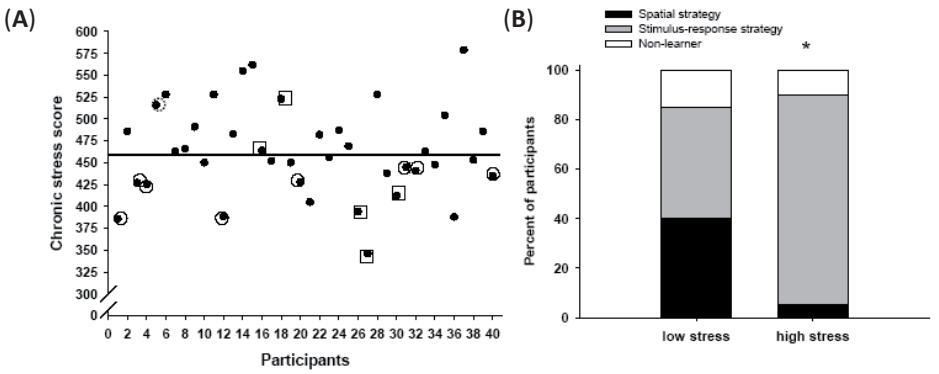

Figure 5

(A) Participants' chronic stress scores as measured by the Trier Inventory of Chronic stress (TICS). According to their chronic stress scores subjects were assigned to the "low chronic stress" and "high chronic stress" groups. The line shows the median. Circle – spatial learner in the "low chronic stress group"; Dotted circle – spatial learner in the "high chronic stress group"; Square – non-learners. **(B)** Percent of spatial, stimulus-response and non-learners in the high and low chronic stress groups. Significantly more participants of the "high chronic stress" group used of the stimulus-response strategy. * $p < 0.05$.

There was no effect of sex on the used strategy ($\chi^2(1)=0.47$, $p = 0.49$; ratio men to women in percent: spatial strategy – 42 to 58, S-R strategy: 56 to 44). Men and women were comparable with respect to their chronic stress scores ($t(38)=0.66$, $p = 0.52$; mean ± SEM: men – 460 ± 13, women – 470 ± 10).

Chronic stress and learning performance: A mixed design ANOVA on the reaction times during training revealed a significant time effect ($F_{(13,442)}=18.04$, $p < 0.001$), while there was neither an effect of chronic stress ($F_{(1,38)}=0.26$, $p = 0.61$) nor a time × chronic stress interaction ($F_{(13,442)}=0.80$, $p = 0.38$) indicating that the performance of high and low chronic stress groups improved similarly over trials. Reaction times increased from about 2 to 6s in the test trial, but were unaffected by chronic stress ($t(38)=0.11$, $p = 0.91$).

Spatial and S-R learners had comparable learning gradients (no main effect of the applied learning strategy ($F_{(1,33)}=0.45$, $p = 0.51$) nor an interaction of time and strategy ($F_{(2,52)}=0.98$, $p = 0.37$).

Verbal report: All participants that were classified as "learner" described the applied strategy in line with the chosen field. S-R learners reported that they used the stimulus (letter M) to identify the win-field; spatial learners described the use of the spatial arrangement (field in the second row of the right column). Non-learners stated that the position of the win-field was completely random and that there was no consistency. Interestingly, S-R learners tended to be more certain that the chosen field is the win-field than spatial learners (mean certainty: S-R 56%; spatial 44%; $t(33)=1.68$, $p = 0.11$).

Discussion

Our results showed that the experience of prolonged or repeated stress in mice and humans affects the learning strategy (S-R or spatial) used to acquire a task. (1) Repeated exposure to rats increased the use of an S-R strategy in mice. (2) Experiencing relatively high levels of stress within the three months prior to testing were associated with a significant change in the used learning strategy (derived from test trial performance and confirmed by subjects' verbal reports) towards more S-R learning in healthy young men and women. These effects refer to a change in the quality of learning.

Previous studies demonstrated that acute stress modulates multiple memory systems in rodents and humans in a manner which favors S-R over spatial learning and memory (Kim et al. 2001; Packard and Wingard 2004; Schwabe et al. 2007). Impairing

effects of chronic stress on hippocampus-dependent forms of learning and memory are well known (Bodnoff et al., 1995, Kleen et al., 2006, Wright & Conrad, 2005) and parallel changes in hippocampal plasticity (Bodnoff et al. 1995; McEwen 1999a; Conrad 2006). Indications that chronic stress affects learning strategies are derived from three studies (Grootendorst et al. 2001; Wright and Conrad 2005).

Grootendorst and colleagues (Grootendorst et al. 2001b) used the same paradigm of rat stress as we did and reported impaired spatial learning in the circular hole board task in 6 month old wild type mice with a C57BL/6J background. The training protocol of the circular hole board task covered several days, followed by a free exploration trial to detect search strategies. Remarkable was the shift to more perservative strategies, i.e., repeatedly return to the same hole, in the rat-stressed group. The same rat stress paradigm also impaired spatial learning in the Morris water maze together with a shift in search strategies from predominantly persistent in controls (60%) to concentric (58%) in rat-stressed mice (Grootendorst et al., 2001a). Both studies indicate that different learning strategies might have been used during training sessions, while the present study demonstrates that chronic stress indeed alters the learning strategy used to solve the task.

The findings of Wright and Conrad (Wright and Conrad 2005) pointed to an intriguing interaction of environmental conditions and task performance. Whereas chronically stressed rats were impaired in a Y-maze task which required the use of extramaze cues, i.e., hippocampus-dependent spatial learning, the introduction of intramaze cues eliminated the impairment. Thus, providing the use of more than one approach to solve the task allows switching to other problem-solving strategies. We conclude that their, like our task allowed for caudate-based stimulus-associated learning in addition to spatial learning, thereby rescuing performance (i.e., quantitative learning parameters). Our experimental setup clearly revealed the use of distinct learning strategies as a consequence of chronic stress.

Moreover, our data support the view of a non-competitive, cooperative interaction between memory systems (Voermans et al. 2004). It could be argued that chronic stress induced changes in the morphology of neurons decreases the functionality of the hippocampus (McKittrick et al. 2000; Fuchs et al. 2006), and therefore, the caudate nucleus might compensate for hippocampal impairment. This is not necessarily a case of the caudate “out-competing” the hippocampus but could be seen as the two systems working in parallel and one taking control when the other is dysfunctional.

Twenty-four hours after training, behavior of chronically stressed and control mice differed both qualitatively and quantitatively. Stressed mice behaved less

predictably than controls, in that they more often chose a different hole during the first trial of day 2 than on the test trial the day before. Whether this is due to chronic stress effects on memory consolidation or retrieval can not be decided here. To disentangle consolidation and retrieval effects, stress has to be administered either within a certain time window after learning or immediately prior to retention testing. Obviously, this is impossible in chronic stress studies. Next to differences in behavioral consistency, we obtained group differences in performance 24hrs after training. Now, stressed mice appear to perform "better", based on latencies and hole visits than mice of the control group. Does this indicate superior memory in chronically stressed animals? In our view, it does not. Memory effects would be expected especially in trial 1. Yet, group differences were absent in trial 1 but increased in the second and third trial. It is more likely and also suggested by others that chronic stress attenuates rodents' motivation to explore (Tejani-Butt et al. 1994; Conrad et al. 1999). We propose that performance 24hrs after training presents motivational rather than memory effects of chronic stress.

Moreover, chronically stressed mice moved significantly faster than controls which might suggest higher emotionality after chronic stress. Long-lasting effects of repeated stress on predominantly fear-related behavior and characteristic exploration patterns have been found in rodents (Grootendorst et al. 2001b; Wood et al. 2008) and humans (Armony et al. 2005). Importantly, others describe these manifestations of enhanced emotionality in relation to stress-induced structural alterations in hippocampus and amygdala. While chronic stress induces dendritic atrophy and debranching in hippocampal neurons, it enhances dendritic arborization and synaptic connectivity in the amygdala (Vyas et al. 2002; Mitra et al. 2005). Interestingly, the amygdala has been assigned a critical role in acute stress effects on memory functions (Kim et al. 2001; Roozendaal 2002) and in the "emotional" modulation of spatial and S-R learning (Packard and Wingard 2004). Intra-amygdala infusions of anxiogenic drugs were sufficient to switch learning strategies form predominant spatial to more S-R learning in rats. It is tempting to speculate that the amygdala plays also a critical role in the observed modulation of spatial and S-R learning by chronic stress.

Corroborating previous rodent and human studies we obtained no differences in quantitative learning parameters between spatial and S-R learners during task acquisition, neither in humans nor in mice (Kim et al. 2001; Schwabe et al. 2007). However, 24hrs later S-R learners showed decreasing latencies, number of holes visited and distances moved over the three trials on day 2, whereas all these parameters were increased in the spatial learners in the stress group - same as in spatial learners in the control group. If longer latencies in the second and third trial are indicative for motivation to explore

which in turn is - as argued above - attenuated by chronic stress, then the differences between spatial and S-R learners on day 2 might be interpreted as indication of a higher chronic stress level in S-R learners.

A challenging question derives from the fact that a certain percentage of the tested population of both species is resistant or vulnerable to the effects of stress. Here, the contribution of an epigenetic predisposition could be tested in animals experiencing discrete early life events like maternal care (Meaney et al. 2007). Additionally, assessing the degree of emotionality which is known to modulate cognitive performance (Packard and Wingard 2004; Brinks et al. 2007a) could contribute to the understanding of a resistant or vulnerable phenotype.

Chronic stress has been frequently associated with “depressive-like” symptoms (for reviews: Willner 1997a; Blackburn-Munro and Blackburn-Munro 2001). Here, the focus was primarily on emotional and motivational factors. Several authors showed that chronic stress contributes to anhedonia (the core symptom of the melancholic subtype of major depression) expressed e.g., as reduced sucrose consumption and preference or reduced sexual behavior in rats (Konkle et al. 2003; Gronli et al. 2005). In the present study, we demonstrate that chronic stress leads to a shift from elaborate “cognitive” to rather rigid “habit” learning. Comparable cognitive dysfunctions were observed in depressive patients. For instance, Harvey and colleagues (Harvey et al. 2004) as well as Purcell and colleagues (Purcell et al. 1997) report deficits in mental set shifting in patients with depression. We suggest that cognitive rigidity, here expressed by the S-R learning strategy, is an important factor in the etiopathogenesis of depression.

Finally, some limitations of the present study have to be addressed. The human task we used here is relatively simple and it is rather unlikely that it is dependent on the hippocampus *per se.* Memory for a single location is primarily a function of the parahippoacmpal cortex (Duzel et al. 2003). Alternatively, choosing of the win-field could be done using a simple S-R strategy without making use of any external landmarks. Thus, task difficulty might be an even more contributing factor rather than the fact that a task is hippocampus-dependent or not. Furthermore, we compared in the present study effects of experimentally induced chronic stress (mice) and self-reported stress (humans) which might raise questions regarding the comparability of the chronic stress effects in mice and man. This is a problem hardly to solve because chronic stress cannot be induced experimentally in humans, for obvious reasons.

Moreover, it is important to note that we did not examine effects of severe, pathological stress. Human subjects were healthy. Chronic stress levels were rather moderate. We stressed mice for 11hrs over a period of two weeks. In line with the study

of Grootendorst et al. (2001a) this resulted in increased basal corticosterone secretion indicative for an effective stress procedure. One of the very few studies that varied the duration of chronic stress found a biphasic effect on performance in a radial maze task. While 21 days of stress resulted in memory impairments, 13 days of stress did not impair but even enhanced memory performance (Luine 2002). It is likely that our "rat stress" paradigm belongs to the category of rather mild chronic stress that still allows adaptation and prevents performance impairment. Extending the stress period in mice and testing a patients suffering from a stress-related disease will provide answers to the more detrimental effects of chronic stress. Initially, chronic stress-induced changes should be viewed as signs of an adaptive response, yet the potential for damage and pathology is increased.

So far, research on memory effects of chronic stress predominantly focused on quantitative parameters such as the number of items remembered in humans and latencies to a goal in animals, i.e. *how much* is learned. The present findings show clearly that chronic stress affects the quality of learning; i.e. which memory system is involved in the process of learning, *how* an individual learns. Independent of the used memory system, quantitative parameters may remain unchanged and thus veil the actual effects of stress on learning and memory. The use of S-R instead of spatial strategies appear to be a first signal of the impact of chronic stress in a vulnerable individual, while the level of performance can still be maintained, as long as the environment remains stable (such as during the training trials in the present studies) and alternative approaches are allowed.

Acknowledgements

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Chapter 9

General Discussion

Outline

9.1. Introduction

For decades, treatment of depression has relied on combined behavioral and neuropharmaceutical approaches, which usually take weeks to become effective. These approaches invariably are aimed to reduce the negative symptoms (including anhedonia, a key symptom in depression) patients experience in their daily activities they previously found enjoyable. Still, faster acting and more efficient drugs are needed. The chronic inability to cope with stress is a major risk factor in the precipitation of depression. Hence, there has always been a line of reasoning that intervention in stress system activity *per se* could be a more direct therapeutical approach towards reduction of depressive symptoms. Following, the glucocorticoid antagonist mifepristone (RU38486=MIF) was tested for its efficacy in depressive patients. MIF appeared to be a rapid acting drug primarily abolishing the psychotic symptoms characteristic for a subtype of depression, i.e. psychotic major depression (DeBattista and Belanoff 2006 and the discussion by Carroll and Rubin 2006), while also ameliorating other negative symptoms of depression such as emotional dysregulation, mood disturbance and anhedonia.

The **objective** of the research described in this thesis was to develop a mouse model of depression that would express anhedonia, induced by chronic stress. Anhedonia was assessed by studying the behavioral response to positive stimuli, and reward expectation. We hypothesized that a chronically stressed individual would have a reduced response to such positive stimuli. To test this hypothesis we have exposed mice repeatedly to a psychosocial stressor using our 'rat stress' paradigm. Furthermore, we explored the impact of reward on behavioral performance of the 'depressed' mouse. As a potential therapeutical approach we assessed the outcome of repeated pharmacological blockade of glucocorticoid receptor (GR) function using MIF on stress system activation and behavioral responses.

First we designed a novel method of drug administration to mice, and optimized several behavioral tests. Next, the expression of anhedonia was validated by phenotyping the consequences of chronic stress in our model. Also the outcome of repeated GR blockade with the GR antagonist MIF was examined on neuroendocrine and behavioral response patterns. The last step aimed to compare the impact of chronic stress in healthy human volunteers with its effects in naïve mice on two distinct learning and memory systems.

9.2. Methodology

Drug delivery of glucocorticoid ligands

We deduced the magnitude of the stress response from the enhanced and long-lasting elevations of corticosterone (CORT) secreted by the adrenals. CORT is the endproduct of the HPA axis and feeds back on the brain to modulate the processing of information underlying the neuroendocrine and behavioral response pattern. This action exerted by CORT is mediated by MR (mineralocorticoid-) and GR activation that operate in complementary fashion (Oitzl and de Kloet 1992; De Kloet et al. 1998).

Most often, drug delivery involves hand-restraint administration (e.g., subcutaneous, intraperitoneal or by gavage), which leads to a concomitant, uncontrolled, and unwanted activation of the stress system (Balcombe et al. 2004). This unwanted stress effect would be a serious confounder in our experiments. Therefore, a method of stress-free drug delivery was required. Using oats, we succeeded in designing a novel non-invasive, stress-free method of glucocorticoid administration in mice (**Chapter 3).**

Drug delivery via oats reduced stress system activation as is evident from the following consideration: The method of drug delivery is non-invasive and avoids handling of the mice; they can remain in the home cage (= non-invasive) where the oats are delivered in a separate feeding cup. Although this procedure elicits a short-lasting, slight increase in plasma CORT secretion, its magnitude is not at all comparable to injection-induced effects, neither in quantity nor quality. Removal of physical contact between human and mice also has the advantage that minimal training is required to deliver the drug of choice to the animal; there is no restraint and no injection needed. Hence, the oat procedure minimizes variability of drug effects induced by the experimenter. Animal discomfort is also strongly reduced due to refinement of the method. Moreover, oats containing the glucocorticoid ligand(s) can be offered during both the light and dark period of the 24h circadian cycle. Because oats were given in addition to standard food and water regimes, the mice considered the oats as a treat. This was evident from the observation that all mice readily ate the oats within 10 min after administration. As a result, the need for food and/or water deprivation, which is considered to be stressful to mice, was by-passed (Sommerville et al. 1988; Duclos et al. 2005). We have shown that mice eat the oats with and without glucocorticoid ligands, for at least 7 consecutive days (**Chapter 4**).

The preferred method of administration depends on the experimental design. Drug delivery via oats is not selective as it reaches the entire body. Circulating CORT

has been shown to cross the blood-brain-barrier, although the concentrations in blood plasma vs. brain tissue differ. Penetration of MIF through the blood-brain-barrier is hampered and the drug is rapidly metabolized albeit in active metabolites. Therefore, high concentrations of the antagonist are to be administered (Karssen 2003). Collectively, GR and MR agonists and antagonists delivered via oats likely can activate and block MR and GR functions in brain.

Drug delivery via oats in close-context with behavioral testing is also feasible. However, it might be less suitable to study fast drug effects. The use of oats as a reward in close-context with learning and memory testing might be confounded however, by the postprandial increase in blood glucose, resulting from oats consumption. Glucose is known to modulate cognitive functions (Messier 2004) and can modulate glucocorticoid action (Gagliardino et al. 1984; Peters et al. 2004). However, the observed glucose increase following oat consumption is a mere fraction compared to the glucose concentrations that are required to modulate learning and memory processes in which glucocorticoids are involved (Gold 1986; Messier 2004; Dalm et al. 2009b). Therefore, a role of postprandial glucose is unlikely.

Drug-delivery via oats requires that mice are single housed, at least during the time of drug delivery. In the majority of behavioral tasks the animals are phenotyped one at a time. For experimental designs that require animals to be treated while group-housed, partitioning of the home cage might be a possibility. However, it is likely that interference with the home cage environment will introduce an additional stress factor (Ouagazzal et al. 2003; Chourbaji et al. 2005) to which the animals can be habituated by a handling procedure.

Advantages and disadvantages of drug delivery via oats are summarized in Table 1 below:

Table 1: Advantages vs. disadvantages using drug delivery via oats.

Advantages	Disadvantages
Non-invasive	Time required for the mice to eat the oats is 10 min at a minimum
Minimal corticosterone secretion during delivery, i.e. stress-free	Animals single-housed
Reduces animal's discomfort	
Can be used by any experimenter without extensive training in animal handling	
Administration can be continued for at least 7 days	
Application of drugs in behavioral context	
Application possible throughout the day	
Self-administration resembling human drug delivery	

In conclusion, drug delivery via oats reduces unwanted stress system activation and can be used in close-context with learning and memory tasks. The preferred method of drug delivery will depend on the scientific question addressed and subsequent experimental design.

9.3. Learning and memory reinforcement

Decades ago, Huston and colleagues presented a memory processing theory of reinforcement, and proposed that the reinforcer acts on a memory of either the response or the stimulus-response contiguity (Huston et al. 1974; Huston and Mondadori 1977). It has provided a framework for studies that have demonstrated a close correspondence between memory promoting and reinforcing effects of natural reinforcers like food (Huston and Oitzl 1989). We demonstrated that post-training reward (access to sugar) in close context with learning facilitates spatial memory performance in mice (**Chapter 5**).

Long-term memory was improved by sugar-reward in both spatial tasks, expressed as superior performance in the first trial of the following day. Whereas the memory facilitating effect in the circular hole board was observed already after the first contingency -location of and moving through the exit hole and sugar consumption, it took several days until it was obvious in the Morris water maze. This time-related effect of the reinforcer is most likely due to task-inherent properties (i.e., aversiveness, stress system activity, testing environment). However, common to both tasks is that goal-directed behavior during the training trials and the persistence of the search pattern in the area of the platform and exit hole are strengthened. General activity and velocity as behavioral responses to the task were not reinforced. Thus, the memory trace of how to locate the platform or exit hole is strengthened by the sugar reward. This memory facilitating effect of sugar is most obvious reinforced in the earlier phases of learning.

In line with other viewpoints (Whishaw 1995; Wotjak 2004), we consider the circular hole board test a procedure that is better adapted to the species-specific needs of mice. Moreover, the circular hole board task allows to collect a broader set of variables related to motivation and emotional expression than our water maze paradigm. The limited number of training trials in the circular hole board provides an opportunity to implement pharmacological interventions in close-context with training events (see Table 2 for an overview of circular hole board vs. water maze).

Table 2: Characteristics of the Circular hole board and the Morris water maze

Characteristic	**Circular hole board**	**Morris water maze**
Environment	Dry	Wet
Motivation	Appetive	Aversive
HPA activity	Low CORT	High CORT
Training trials	2 trials/day	4-5 trials/day
Predictive	2nd trial always shorter latency	High Trial-trial variability
Learning and memory	Short- and long term memory	Short- and long term memory
Anxiety measurable	Yes	No
Pharmacological intervention	Can be implemented before and after each trial seperately	Can be implemented following a set of trials

Effects on consolidation processes were achieved by allowing mice free access to sugar in the home cage after the last training trial of the day. As expected, a sugar reward in close-context with training (immediately, but not 4hrs later) facilitated memory in both spatial tasks, albeit within different time domains.

Studies on the effect of sugar reward and other drugs on learning processes include handling, restraining and injecting the animal and thereby, additionally increasing stress-hormone secretion (Meijer et al. 2006). The task-independent activation of the stress system by these manipulations may contribute to the modulation of memory processes. By giving the mice free access to sugar in close context with their performance in the learning task, we have introduced a non-invasive method for sugar reward that is devoid of possible interfering effects of stress hormones on memory processes.

Concluding, in line with the concept by Huston & Oitzl (Huston and Oitzl 1989) post-training sugar in close context with learning facilitates spatial memory in mice, via modulation of consolidation processes. We have utilized this method to study the effect of chronic stress in reward processing in **Chapter 7**.

9.4. Conceptualization of our animal model of depression

We validated our 'rat stress' animal model for depression by three criteria (Willner 1984): (1) *construct validity*: the model mimics the etiology of depression; (2) *face validity:* the model replicates a number of symptoms characteristic for depression; (3) *predictive validity*: treatment of symptoms has identical effects in the mouse model as in humans.

9.4.1. Construct validity

Depression is characterized by disturbances in emotional and cognitive processes, together with a dysregulated circadian and stress-induced secretion of glucocorticoid hormones. The expression of these symptoms may be of genetic- or environmental origin or a combination of both, and can be modulated by cognitive and non-cognitive inputs (Bale et al. 2010; Mann and Currier 2010).

Chronic stress is considered a vulnerability factor for the development of depression (De Kloet et al. 1998; de Kloet et al. 2005; McEwen 2005). Whereas all kinds of stressors induce behavioral alterations and concomitant changes in the regulation of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Endo and Shiraki 2000; Anisman and Matheson 2005), psychological stressors are ethologically relevant and resemble the kind of stress that is related to depression in humans (Calvo-Torrent et al. 1999; Apfelbach et al. 2005; Beekman et al. 2005). Central features of chronic psychological stressors in humans are repeated, unpredictable and uncontrollable exposure to (or imagination of) threatening situations. To mimic such conditions of chronic stress, animal models make use of confrontations with territorial conspecifics and exposure to predators with or without physical confrontation (Apfelbach et al. 2005).

Previously, our group generated a “chronic stress mouse model” by exposing mice repeatedly to the presence of a rat, a procedure referred to as chronic ’rat stress’. Mice and rats could hear, see and smell each other, while preventing physical contact (Grootendorst et al. 2001b). Therefore, we expected that a psychological stressor would target the prefrontal cortex, amygdala and hippocampus. These brain areas show altered synaptic plasticity in rodents exposed to psychological stressors (Diamond and Park 2000; Diamond et al. 2006) and in patients that suffer from depression (Drevets et al. 2008); antidepressants affect this synaptic plasticity (Vouimba et al. 2006). All three brain areas express high levels of GR, indicating a high sensitivity to glucocorticoids secreted during stress (Reul and de Kloet 1985). Although we did not measure synaptic plasticity in mice exposed to our chronic stress paradigm, the observed acute and long term psychoneuroendocrine effects were evident and not detectable in non-stressed mice. These psychoneuroendocrine effects will be discussed in detail in the section related to *face validity*.

We used 3 months old male C57BL/6J mice for all the studies on the animal model described in this thesis for several reasons. First, the C57BL/6J mice is most commonly used in generating transgenic mice, including those with targeted MR and GR expression alterations (Muller et al. 2002; Urani and Gass 2003; Kolber et al. 2008). Hence, this

mouse strain has been extensively phenotyped (i.e., neuroendocrine, emotional and cognitive processes). Secondly, 3 months old mice are regarded as late adolescent or young adults, a period in their lives where brain development is still ongoing. Thirdly, the age of onset of mood/affective disorders has been shown to occur in the median age range of 25-45 years (Kessler et al. 2007). Finally, this age group displays a 24h circadian cycle of CORT secretion which approximates human cortisol secretion during the day (Krieger et al. 1971; Steiger 2003), taking into account the fact that mice are nocturnal animals. The cycle is characterized by peak concentrations of CORT at the onset of darkness, which is the active period for mice. Thereafter, CORT concentrations gradually decrease during the remainder of the dark and into the light period of the circadian cycle (**Chapters 2** and **4**).

Concluding, construct validity of our animal model of depression was achieved by exposing young adult male mice to 'rat stress' which elicits an etiological relevant type of psychological stressor.

9.4.1.1. Stress system activity and multiple memory systems in mice and men

As described in **Chapter 7**, stressed mice reached the same level of performance as non-stressed mice to locate the exit hole in the complex environment of the circular hole board. Interestingly, during the free exploration trial following the training trials, stressed mice preferred using a perservative search strategy, which is less efficient then a serial search strategy (Grootendorst et al. 2001a; Grootendorst et al. 2001b). It was recently discovered that stress may operate as switch between multiple memory systems (White and McDonald 2002; Schwabe et al. 2010). Neurobiological studies demonstrate that memory is organized in multiple brain systems. These memory systems differ with respect to the kind of information they process (Gabrieli 1998; Squire 2004a). Spatial hippocampus-dependent memory supports the acquisition of flexible, consciously accessible knowledge (Scoville and Milner 1957; Eichenbaum 2004). Non-spatial, stimulus-response (S-R) learning processes associations, such as ''stop your car when the traffic lights are red". It is not necessarily accessible to consciousness and relies on the caudate nucleus (Knowlton et al. 1996; Jog et al. 1999).

Whereas the circular hole board is considered a spatial memory task, the setup of the circular hole board experiment described in **Chapter 7**, did not allow to study the use of the two different memory systems that might be used by chronically stressed and non-stressed mice to solve the task. Therefore, we created a learning and memory paradigm for the circular hole board (**Chapter 8**), based on a study performed by Kim et

al (Kim et al. 2001). The same principle was used for the human variant of the learning task. This allowed a translational approach to study the impact of stress on the use of memory systems between mice and human.

Our results show that a period of chronic stress in mice and humans switches the use of either a spatial or S-R learning strategy, in favour of the latter. The observed learning performance refers to a change in the quality of learning, rather than in the quantity of learning (Schwabe et al. 2010).

Acute stress prior to training in a task that could be acquired by a hippocampus-based spatial, and a caudate-based S-R strategy resulted in predominantly caudate-based learning both in rodents and humans (Kim et al. 2001; Packard and Wingard 2004; Schwabe et al. 2007). We show that chronic stress has the same effect. Going back to the results we previously described (Grootendorst et al. 2001a; Grootendorst et al. 2001b, **Chapter 7**: thesis), it is likely that our chronic stress paradigm induces a switch from using S-R over the spatial learning strategy, in both the water maze and circular hole board.

The stress-induced modulation of hippocampus-dependent and caudate-dependent systems is assumed to be influenced by the amygdala (Packard and Wingard 2004). Emotional components like anxiety, punishment, reward, are part of the majority of behavioral tasks for rodents, including the water maze and circular hole board (Dalm et al. 2009b; see **Chapter 5**). The associated stress increases the excitatory amygdala input to the hypothalamus PVN producing enhanced release of CRH and ultimately a larger output of CORT. This additional increase of CORT could affect both the quantity and quality of cognitive performance. Whereas learning is critical for adaptation to the environment, when the adaptation is inappropriate, it can also produce dysfunctional patters of thinking and emotional responding (Schwabe et al. 2010). In fact, heightened activity of the amygdala improves memory consolidation of negative events (Roozendaal et al. 2009).

As a result of stress an organism may switch from perceiving the world from a balanced positive and negative perspective, towards a more negative perception, due to increased use of an S-R learning strategy that maintains focus on threats (negative) rather than reward (positive). Interestingly, a cognitive framework for depression suggests that positive mood promotes associative processes and vice versa (Bar 2009). From an evolutionary standpoint this would be of value as it allows the organism to learn and explore multiple alternatives that a given environment provides regarding coping with rewarding and aversive stimuli. The recent focus on the impact of stress on multiple memory systems actually supports this cognitive framework. Of course, perception

of the environment involves multiple brain regions to work in concert resulting in an adaptive response, either beneficial or maladaptive, but is always aimed to restore homeostasis (McEwen 2000).

Concluding, we demonstrate that chronic stress leads to a shift from elaborate "cognitive" spatial to rather "rigid" S-R/habit learning. Comparable cognitive shifts were observed in patients that suffer from depression (Purcell et al. 1997; Harvey et al. 2004). We suggest that cognitive rigidity, expressed as favoring S-R learning strategy used for problem solving, is an important factor in the etiology of stress-related affective disorders including depression.

9.4.2. Face validity

Affective disorders like psychotic, major and bipolar depression, share several characteristics (de Kloet et al. 2005): emotional changes related to approach or avoidance behavior, loss of interest or pleasure in daily activities, impairment of cognitive functions, reduced motor activity and alterations in the circadian pattern of physiological, neuroendocrine and behavioral responses (Endo and Shiraki 2000; Volkers et al. 2002; Keller et al. 2006). Our chronic stress model was evaluated based on the degree of symptoms expressed as described above; see Table 3 for an overview.

We particularly focused on the expression of anhedonia using different methods aimed at detecting a disturbance in reward processing i.e., reduced responsiveness to rewarding stimuli. Loss of responsiveness to rewarding stimuli indicates a shift in the detection, interpretation and response to negative and positive stimuli that are part of the external world of the organism. Stress has been shown to alter the perception of those stimuli via modulation of stress hormone receptors in the brain, in particular the hippocampus (Oitzl and de Kloet 1992). Hence, we expected that chronically stressed mice would have an altered perception of the environment. To test this, we determined: 1) the behavioral response in a novel environment; 2) modulation of learning and memory processes by reward; 3) sucrose consumption and preference.

9.4.2.1. Anhedonia: behavioral inhibition in a novel environment

For patients that suffer from depression, the negative effects exist in both familiar and novel environments (Volkers et al. 2002; Keller et al. 2006). Chronic 'rat stress' alters the perception of a novel environment as indicated by distinct behavioral response

Table 3: Symptoms associated with depression in humans, and reference to the Chapters in thesis that assessed the expression of the human-like symptoms in our mouse model of depression.

Symptoms of depression *	Assessed in Chapter	Symptoms expressed	Parameter(s) for stressed mice
Anhedonia	6 and 7	Yes	Behavioral inhibition Altered response towards positive stimuli
Appetite/Weight changes	6	Yes	Bodyweight fluctuated
Sleep disturbances/ circadian activity pattern	6 and 7	Yes	Daily organization of behavior in the home cage was different
Psychomotor retardation	6 and 7	Yes	Behavioral inhibition during novelty exposure
Fatigue/loss of energy	not assessed	n.a.	n.a.
Depressed mood	No	n.a.	n.a.
Feelings of worthlessness/guilt	No	n.a.	n.a.
Diminished ability to think/make decisions	6, 7 and 8	Yes	Shift to a more rigid search strategy, likely triggered by an altered perception; sugar reward partially improved memory
Thoughts of death/ suicide	No	n.a.	n.a.

**Source: Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV, American Psychiatric Association 1994.*

patterns, similarly as has been described previously (Grootendorst et al. 2001b). We extended the characterization of behavior to the familiar environment of the home cage of individually stressed, and non-stressed mice. Long-term automatic recordings using the PhenoTyper observation cage (Noldus Information & Technology BV, Wageningen, The Netherlands), revealed a reduction in general activity, and overall, a disturbance in the daily organization of behavior (**Chapter 6**). Most pronounced was the expression of behavioral inhibition measured as a delayed onset to explore the environment.

Animals need to forage for food to meet their energy demands, while at the same time minimizing the risk of being exposed to a life-threatening situation, i.e., a predator or the threat of a possible predator (Lima and Bednekoff 1999). Although mice were exposed to the 'rat stress' outside their home cage, they displayed inhibition of exploration together with focused attention upon return to their home cage. This indicates that the impact of the rat exposure *outside* the home cage was carried over into the situation of the home

cage where the actual exposure to the rat never occurred. The inhibition and the reduced exploratory activity in the home cage has an adaptive value as it temporarily decreases the risk of predation (Nordahl and Korpimaki 1998), even though the actual danger is no longer present. Interestingly, the behavioral inhibition and reduced exploratory activity in the home cage is maintained if the mouse is placed in a novel cage (**Chapters 6, 7** and **8**).

In the novel environment of the circular hole board, stressed mice alternated between serial (sequential hole visits) and perservative (repetitive visits of the same hole) search strategies more often than controls. Exploration of the holes and the rim of the board are important to locate possible routes of escape from the open, unprotected environment. We observed that stressed mice were slower in starting to visit holes and performing rim dips. We also observed changes in head-dipping behavior which have been associated with altered information processing, and therefore may reflect the anxiogenic or anxiolytic state of the mouse (File and Wardill 1975b; File and Wardill 1975a; Takeda et al. 1998). In addition, the anxiolytic state was observed for at least 3 months after cessation of the stressor (Grootendorst et al. 2001b), when mice were tested in the light-dark box. Thus, independent of the environmental context (circular hole board vs. light-dark box), stressed mice displayed behavioral inhibition, indicative for more anxiety-related behavior. Apparently, the behavioral inhibition became part of the daily organization of behavior and lasted weeks after cessation of the stressor (**Chapters 6** and **7**).

Exploration is considered a self-rewarding behavior. In the early 1980's Katz and colleagues studied the effects of stress on open field (novelty exploration) behavior. A history of chronic stress exposure reduced the novelty induced activity, which could be reversed by chronic antidepressant treatment (Katz and Hersh 1981; Katz et al. 1981; Roth and Katz 1981). Whereas the inhibition of exploration is generally related to anxiety, it might also indicate the loss of hedonic responses, as suggested by Bevins and colleagues (Bevins and Besheer 2005). Thus, the exploration patterns in a familiar or novel environment might provide leads to the emotional state of the animal (File 2001; Kalueff et al. 2006).

We showed that chronic 'rat stress' altered the daily organization of behavior such that situations were perceived as threatening and negative, rather than neutral or positive, both in a familiar and novel environment. The chronic stress forced mice into a conflict situation, i.e. approach vs. avoidance, where they have to maintain food and water intake, while lowering the risk of being predated. In 1976, Gray conceptualized two motivation

systems that act in response to environmental stimuli: (i) the Behavioral Activation System (BAS), that controls approach behavior in response to cues of reward via dopaminergic activity in the mesolimbic system; (ii) the Behavioral Inhibition System (BIS), that is sensitive to cues of threat and controls inhibition of behavior via noradrenergic and serotonergic activity in the limbic septohippocampal system and amygdala (Gray 1976; Gray 1987; Gray 1994). Chronically stressed mice in our model displayed increased inhibition of behavior focusing primarily on threats in the environment. Thus, it is likely that the BIS has become more dominant. In fact, this has been shown for patients that suffer from depression (Kash et al. 2002) and schizophrenia (Scholten et al. 2006).

Concluding, chronic stress changes the perception of the environment by focusing on stimuli that might indicate a threat at the expense of possible rewarding stimuli. This in turn shifts the sensitivity between the two proposed motivational systems from the dopaminergic BAS towards a more sensitive limbic BIS. This sensitivity becomes, at least for weeks, embedded into the daily organization of behavior.

9.4.2.2. Anhedonia: modulation of learning and memory processes by reward

The impact of stress on learning and memory processes is described as impairing, improving or ineffective. The key towards disentangling these apparent paradoxical effects exerted by stress is the timing and context of stressor exposure in relation to the learning and memory processes (Joels et al. 2006; de Quervain et al. 2009; Conrad 2010). Glucocorticoids are often examined in this respect as representing 'stress', but obviously these hormones operate in concert with all other stress mediators, and this interaction between the various stress signals adds another level of complexity. Yet, glucocorticoids target the hippocampus, which is considered a key brain structure in the processing of novel information.

The hippocampus functions at the crossroad where novel information is detected, evaluated and appraised. Glucocorticoids operate in the hippocampus through MR and GR, which are implicated during information processing. In fact, these receptors mediate the CORT effects on the appraisal processes of novel information, as well as on memory storage. Additionally, CORT also acts on other circuits impinging on the hippocampal formation, notably the amygdala-entorhinal input through which emotions are regulated. It is well known that the more emotional an experience is, the better it is remembered, and glucocorticoids acting through MR and GR in the hippocampus have a key role in labeling the emotions in space and time (Oitzl and de Kloet 1992; McGaugh and Roozendaal 2002; Brinks et al. 2007c).

Indeed, long term exposure to glucocorticoids or stress has been shown to alter synaptic plasticity in hippocampus in a spatial context, in prefrontal cortex with respect to response selection and in amygdala regarding the emotional value of stimuli, amongst other brain structures, affecting information processing (de Kloet et al. 1999; McEwen 1999b; Mizoguchi et al. 2000; Roozendaal et al. 2009; Conrad 2010). Interestingly, human fMRI studies show that joint activation of hippocampus and brain reward regions, involving dopamine, is crucial for the development of long term memories (Schott et al. 2006b; Wittmann et al. 2007). Changes in the reward processing system belong to the main symptoms of depression (Keedwell et al. 2005; Martin-Soelch 2009). These changes contribute to the expression of anhedonia. We have exploited our method described in **Chapter 5**, to study the impact of chronic stress on the facilitating effect of post-training reward on cognition.

We confirmed and extended our previous results using the chronic 'rat stress' model, and showed that stressed mice displayed a distinct learning curve (Grootendorst et al. 2001b). The stressed mice were slower to locate the exit hole during the first 2 days of acquisition training; thereafter they were as fast as non-stressed mice. Analysis of the free exploration trial after completion of the acquisition learning trials indicated that the stressed mice used a more perservative strategy to locate the exit hole. Controls favored the use of the serial search strategy which became apparent as a pattern resembling a 'see-saw', while the stressed mice showed a smooth though delayed learning curve during training trials. Thus, stress changes the way an animal navigates within a given environment. In fact, we demonstrated in **Chapter 8** that chronic stress produced a shift in the use of search strategies by favoring stimulus response over spatial strategies in mice (Schwabe et al. 2008).

We investigated the impact of stress on reward processing by utilizing our developed method, providing mice post-training free access to 30 mg sugar in close-context with a training trial. As task, we used the circular hole board, which permits multiple readout parameters on emotional and cognitive responses (see **Chapter 5**).

In stressed mice post-training sugar partially restored performance to the level of control mice that did not receive sugar post-training. The graphical representation of the latency to exit hole displayed a similar 'see-saw' pattern (i.e., latency during 1st trail of the day higher than during the second trial). However, controls without-sugar gradually improved performance (latency in trial 1 approximating latency in trial 2), whereas stressed mice that received sugar remained slower during all 1st acquisition trials of the day. This could be due to the overall behavior expressed in stressed mice, namely the initial inhibition which was present during circular hole board training and

up to 1 month later in the light-dark box novelty paradigm. The question then becomes which processes are affected by stress, and which of those are partially restored to function, under these conditions of post-training sugar.

An organism will need to learn to predict which environmental stimuli are biological meaningful, rewarding or aversive. In complex natural environments, like the circular hole board, this requires the integration of different sensory modalities into coherent memories and the coordination of various motor systems. In particular, the brain must learn and store representations of the biological value of appetitive or aversive stimuli, and recall these representations to control adaptive experience-dependent behavior (for example, when to approach or to retreat; Hammer et al. 1997).

The process of integration during learning and memory processes involves 3 stages: acquisition, consolidation and retrieval. During acquisition, both the task inherent proximal and distal environmental cues are perceived by the organism. In the circular hole board spatial memory task, the goal is to locate the accessible exit hole that is connected via a tunnel to the home cage. Because of the complexity of this task, it will take several training trials before the organism understands the 'rule'. It needs to create a map of the environment, and recognize its own position in relation to the exit hole (note: the mouse is placed in a non-transparent cylinder before it is free to explore the circular hole board environment). In addition, it will start to value the predictable access to sugar reward post-training. Analysis of the training trials revealed that path length, walking velocity and time to leave the start center were not affected by post-training sugar during the course of the learning paradigm. The performance however, did improve from trial to trial and from day to day in sugar-rewarded mice. It is likely that this is due to modulation of short- and long-term memory following re-exposure to the circular hole board environment.

Immediately after learning the memory is still in a labile form prior to being fixed or consolidated in a more permanent form (McGaugh and Herz 1972). This implies that during the post-trial, post-training period memory remains susceptible to disruptive or facilitating treatments. Thus, environmental manipulation and changes in circulating CORT concentration or a reward can act during this labile period and affect spatial learning in rats (Sandi et al. 1997; De Kloet et al. 1998; Akirav et al. 2001; Joels et al. 2006), and mice (**Chapter 5**; (Dalm et al. 2009b). Because the sugar-reward was provided post-training and, importantly, in close-context with the learning task, memory consolidation was affected in both stressed and control mice. Although to a different degree, both groups displayed improved performance due to the reward. This raises the question which process(es) are involved in the observed cognitive enhancement.

Several studies have shown that the intake of palatable food (e.g., calorically dense food containing high amounts of carbohydrates or fats) is increased during periods of stress. In fact, the increased intake results in an improved emotional state in humans (Dube et al. 2005) and lowers the cortisol response to a stressful event (Pecoraro et al. 2004; Ulrich-Lai et al. 2007). In our own studies we observed that exposure to the circular hole board increased corticosterone concentrations in mice (see **Chapter 5** discussion). It is speculated that the free access i.e. self administration, to 30 mg sugar corns of the stressed and non-stressed mice during the (pre-)training phase of the circular hole board learning and memory paradigm might have dampened HPA axis activity, resulting in improved spatial performance and emotional state.

As previously mentioned, the impact of CORT on learning and memory is described as impairing, improving or ineffective, and becomes manifest depending on the timing and context of stressor exposure in relation to the learning processes, described as a U-shaped curve (Joels et al. 2006; de Quervain et al. 2009; Conrad 2010). Previously we showed that chronic 'rat stress' increased basal CORT concentration 7 days after the last rat exposure (Grootendorst et al. 2001b). We did not measure the CORT response induced by circular hole board exposure in stressed and non- stressed mice that had received 30 mg sugar prior. However, as other studies do suggest, daily access to palatable food attenuates the stress response (Ulrich-Lai et al. 2007). Moreover, the intake of palatable drink as stress relief also affects reward pathways and metabolic circuitry in the brain (Ulrich-Lai et al. 2010; Ulrich-Lai et al. 2011).

In conclusion, as observed in human studies the improved performance in stressed mice by post-training sugar might result from the attenuation of HPA axis activity following this reward, which shifts the CORT value towards the optimal range required for modulation of learning and memory processes.

9.4.2.3. Anhedonia: sucrose consumption and preference

Measurement of sucrose consumption or preference is in widespread use in preclinical psychopharmacology to show an alteration in reward responsiveness induced by stress by a change in hedonic responses. Authors describing animal models of chronic stress generally report a decrease in sucrose consumption as a measure for anhedonia (Katz 1982; Pothion et al. 2004; Strekalova et al. 2004; Anisman and Matheson 2005; Willner 2005). We used a 5% sucrose solution (i.e. dissolved table sugar in water), based on a study by Pothion and colleagues (Pothion et al. 2004). They showed that a sucrose

solution between 4-8% resulted into the highest intake (± 11-16 ml) and preference (± 97-98%) in naïve C57BL/6J male mice.

Prior to the start of the 'rat stress' paradigm, mice showed an impressive consumption of (12 ml) and preference for (85%) the 5% sucrose solution (**Chapters 6** and **7**). Control mice further increased their consumption and preference over the course of the experiment. There was a clear time-dependent pattern in the development of increasing consumption of and preference for sucrose in the control mice which was absent in stressed mice. In contrast, stressed mice consumed less sucrose during the initial phase of the 'rat stress' paradigm; since baseline levels were reached only one day after the cessation of rat exposures. During the stress paradigm the stressed mice spent less time near the bottles than controls, which most likely reduced their fluid intake. However, since the preference of the stressed mice for sucrose did not change during the course of the experiment, and water intake was comparable to controls, we feel confident that 'rat stress' affected the hedonic properties of sucrose. Stressed mice did not increase their preference and consumption alike the controls. Based on the findings described in **Chapter 6,** we may conclude that chronic stress induces anhedonia.

However, the outcome of the sucrose preference task does not necessarily indicate expression or absence of anhedonia, as indicated by reduced sucrose consumption and preference. The results of the sucrose testing in **Chapter 7** showed that mice exposed to the same chronic stress paradigm, consumed more volume of both sucrose and water, 35 days after cessation of the stressor. In fact, we found a stress-induced increase of caloric intake. It is known that glucocorticoids stimulate behaviors that are mediated by the dopaminergic mesolimbic "reward" pathways, and also increase the intake of food with high carbohydrate and fat (see review Dallman et al. 2007), so-called "comfort" food. Consequently, we propose that the rat stress procedure affected the reward system, in a manner that also counteracted to some extent the addictive properties of sucrose (Avena et al. 2008).

The volume overload of 300% due to sucrose drinking most likely affected the body's fluid and energy balance. Sucrose by itself is rich in energy, which is utilized directly, stored in adipose tissue or secreted from the body (Peters et al. 2004). Drinking sucrose might have lowered stress-induced CORT secretion as shown by Bell and colleagues (Bell et al. 2002). Indeed, absolute CORT values in response to rat exposure were lower than measured in previous studies (Grootendorst et al. 2001a; Grootendorst et al. 2001b). Our additional experiment revealed **(Chapter 6**), that the 'rat stress' control procedure (placement in another cage) reduced the nocturnal activity for at least two days, while sucrose overload affected the activity pattern only on the day of consumption. Therefore,

the reduced consumption and lesser preference for sucrose is a distinct feature of the rat stressed mice. Consequently, we conclude that the 'rat stress' procedure affected the reward system.

As described in **Chapter 7,** the initial 24h sucrose testing was followed by measuring the preference for the bottle that previously contained the 5% sucrose solution. Despite the fact that the content of the sucrose bottle was changed to water, stressed mice preferred to drink from that water bottle. This can be seen as a sign of habitual learning. The rigidity in behavior might also suggest reduced extinction learning as a consequence of stress (Brinks et al. 2009; Schwabe and Wolf 2009; Schwabe et al. 2011). Exposure to a psychosocial stressor before training in an instrumental task rendered the participants' behavior insensitive to the change in the value of the food outcomes: i.e., stress led to habit performance at the expense of goal-directed performance in humans (Schwabe and Wolf 2009). This proves that recognizing a change in rewarding values is differentially perceived under stress. Also, chronic social stress enhances habit-based learning in mice (Ferragud et al. 2010). We performed the sucrose testing > 1 month after cessation of the stressor and still, stressed mice displayed the apparent habit-like behavior. This suggests that, next to behavioral inhibition (see section 9.4.2.1.) the use of habit-like responses over goal-directed flexible response prevails in chronically stressed mice. The shift in memory systems and response selection has been discussed in more detail in section 9.4.1.1.

What could have triggered the shift in response strategy in stressed mice? Did they perceive the taste of sugar as highly rewarding, strengthening the memory for this location? It would be of great interest to study the time-dependent effects of chronic stress with respect to stress-induced metabolic changes and food intake.

Overall, we conclude that 'rat stress' model induces the expression of anhedonia in mice, and more generally, that chronic stress alters reward processing, leading to shifts in response strategies. As we focused on responsiveness towards positive stimuli, the shift in response strategy is likely due to an altered perception of the environment. Ultimately, rigidity might occur, making the organism less flexible when faced with new challenges.

9.4.2.4. Dysregulation of HPA axis activity

The circadian cortisol secretion pattern is disturbed in patients that suffer from a mood disorder like depression (Belanoff et al. 2001b; Flores et al. 2006). Although numerous mouse models for a wide range of human stress related mood disorders like depression

have been developed, the circadian secretion pattern of mice has received relative little attention. We showed that naïve male C57BL/6J mice display a circadian rhythm that is affected by age (**Chapter 2**). The secretion of CORT was highest for the 9 months old mice and lower in the 3 months and the 16 months old mice, during the light / inactive period, with no differences in total CORT secretion over the dark / active period. In **Chapter 7** the 24h circadian pattern of glucocorticoid secretion is affected by chronic stress in 3 months old mice. One day after termination of the chronic stress procedure the CORT concentration was not increased. However, 6 days after cessation of the stressor, the CORT concentration during the light inactive period of the day was nearly 1.5 times higher.

Concluding, although this finding is suggestive, clearly more data need to be collected on the HPA reactivity regarding face validity of the neuroendocrine system in our 'rat stress' model for depression.

9.4.3. Predictive validity

In our experiments we have used naïve mice, stressed mice and mice that received the GR antagonist MIF. We have not tested whether the short-lasting treatment did ameliorate stress induced alterations in mice exposed to our 'rat stress' paradigm. We did show that post-training sugar reward partially restored learning and memory performance in stressed mice to the level of non-stressed mice (see section 9.4.2.2. for discussion).

The efficacy of GR antagonism in clinical studies could be due to the following factors: (1) The detrimental effects of high CORT levels via GR activation are prevented by the GR antagonism. On the other hand, (2) as a result of GR blockade and subsequent rise in CORT levels, only the MR becomes strongly activated. The recent discovery of MR being located in the membrane and sensitive to high amounts of CORT, exerting non-genomic actions (see Karst et al. 2005; Joels et al. 2008; Groeneweg et al. 2011; Groeneweg et al. 2012) in addition to the well-known genomic action mediated by MR, opens new avenues to discover MR functions. (3) As the pharmacological action of the GR antagonist wanes, GR becomes activated by the high circulating levels of CORT and via negative feedback, shuts off the CORT secretion. By means of recurrent blockade of GR we might force a pronounced circadian pattern of enhanced CORT secretion followed by its suppression. This effect might be of importance for the development of a new balance and threshold for HPA axis activation. Acting in such a way, drugs that antagonize GR or boost MR are likely candidates for novel antidepressants.

9.4.3.1. Effect of daily mifepristone administration on circadian and stress-induced HPA axis activity.

The secretion of CORT (cortisol and corticosterone in humans and corticosterone in rodents) exhibits a circadian pattern (Windle et al. 1998a; Buckley and Schatzberg 2005; Dalm et al. 2005). In humans dramatic changes in circadian patterns of corticosteroid hormones have been described in aging and also in psychiatric and neurological diseases like depression and Alzheimer's disease (Hatfield et al. 2004; Peeters et al. 2004). These changes in cortisol are linked to resistance in GR-mediated negative feedback (Ribeiro et al. 1993; Heuser et al. 1996; Pariante and Miller 2001).

In response to blockade of GR the circadian CORT secretion was altered (**Chapter 4**). Two hours after a single dose of MIF (200mg/kg RU38486) was administered to naïve mice, the CORT concentration was significantly higher and remained elevated for 14h due to interference with negative feedback at the level of GR. Interestingly, during the 7th day of GR antagonist administration no apparent alteration in circadian CORT secretion was observed. The repeated GR antagonism resulted overall in lower CORT secretion during the light period of the day, compared to controls and acute GR antagonism.

How does this paradoxical effect of GR blockade come about? We reason that by blocking GR mediated negative feedback with the antagonist a long-lasting elevation of CORT occurs which persists beyond the actual blockade of the receptor, since MIF is very rapidly degraded and eliminated (see Karssen 2003). Next, the elevated CORT levels will exert a negative feedback action which persists for many hours suppressing CORT even below baseline at 32h post injection. However, CORT secretion remains highly responsive to stressors at that time, which is also evident from the increased adrenal weight after GR antagonist treatment. Hence, we propose that a renewed GR antagonist administration will each day show a diminished effect on the CORT secretion until the HPA axis does not respond at all anymore at the 7th day of administration; this is what was observed.

Repeated MIF administration to rats for 5 days in a 10 fold lower dose than we did, also caused a reduction in ACTH and CORT release. Moreover, c-fos expression in the prefrontal cortex and amygdala, and decreased expression in hippocampal region (Wulsin et al. 2010) was observed. These data suggest that MIF is enhancing inhibitory and suppressing excitatory inputs to the PVN that collectively may account for downregulation of HPA axis activity as well. This downregulation of HPA axis activity is also further enhanced by CORT action via hippocampal MR suggesting a coordinate control by both MR and GR at longer time intervals after antagonist treatment.

In a study by Bachmann and colleagues (Bachmann et al. 2003) MIF and other more specific GR antagonist, administered for 3 weeks in a dose range comparable with that of Wulsin studie, did not increase hippocampal and pituitary GR mRNA expression. However, prefrontal cortex GR mRNA expression was increased after 3 weeks of MIF treatment. We observed that after the first MIF administration MR expression was downregulated in the hippocamus, but upregulated after the 7th administration, specifically in the hippocampal CA2 cell field. The observation that MR function becomes more prominent after GR antagonism is also evident from the shift in hyperactivity displayed on the circular hole board to a more serial search pattern (Oitzl et al. 1994; Oitzl et al. 1997b).

Also the mode of MIF administration appears important. Episodic administration of very high concentrations of MIF down regulates the HPA axis over a few days. This effect can be prevented by increasing the frequency of MIF administration to twice a day. Such a condition of continuous blockade of central GR's enhances the amplitude in circadian and stress-induced activations probably because of a changed set point of the HPA axis. This increase in feedback resistance caused by the chronic infusion of the GR antagonist follows the theoretical prediction by Walker & Lightman (Walker et al. 2010): any system that has a delay between activation and inhibition has to oscillate. However, in all cases, irrespective of the route and dose of administration, the size and weight of the adrenal cortex is increased suggesting hyperfunction of the adrenals during GR blockade.

Concluding: the above finding in our chronic 'rat stress' model may explain how 'normalization' of aberrant cortisol secretion may occur in patients suffering from psychotic major depression that are treated daily with very high doses of MIF (Belanoff et al. 2001a; Thomson and Craighead 2008). It appears that the dose and mode of administration of the GR antagonist is essential for this downregulation of HPA axis activity. The paradoxical strengthening of negative feedback inhibition of CORT secretion by recurrent administration of MIF is most likely achieved by the integration of MR and GR-mediated effects.

9.5. Concluding remarks

In this thesis, the studies were described with mice that had a history of repeated and unpredictable exposure to rats. Our 'rat stress' paradigm is known to elicit an etiological relevant type of psychological stressor, which we found causing neuroendocrine and behavioral changes in the mouse that show features of human depression. The CORT secretion was altered, while an impaired cognitive performance and a reduced preference for positive rewarding stimuli developed. Accordingly, these behavioral changes induced by the 'rat stress' paradigm display features fulfilling *construct* and *face* validity of a mouse model of depression.

In the cognitive domain, chronic 'rat stress' led to a shift from a spatial learning strategy to a rather rigid stimulus-response strategy. This shift towards so called habit learning reflects a state of cognitive rigidity in problem solving behavior. Moreover, exposure to novelty revealed behavioral inhibition in our chronic stress model. Collectively, the behavioral inhibition, the rigidity and reduced flexibility to novelty, as well as the preference for habit learning present a phenotype that provides leads for translational studies. Central to these translational leads is the behavioral phenotype of our mouse model that signals vulnerability for pathogenesis of human stress-related affective disorders including depression. In **Chapter 8** we report our discovery that this shift from spatial to habit learning and rigidity occurred in both mice and humans, after a history of chronic stress exposure.

Our mouse model of depression demonstrated features predicted by the BIS/BAS theory of the late Jeffrey A. Gray (Gray 1982). This theory points to a reciprocal relationship between the limbic noradrenergic / serotonergic behavioral inhibition system (BIS) and the mesolimbic dopaminergic behavioral approach or activation system (BAS) that corresponds to the conflict between two major personality traits i.e., the conflict between the motivation to avoid fear vs. the desire to approach an award or to anticipate joy and happiness. In particular because chronic stress seemed capable to drive the perception of the mice towards focus on potential threatening rather than rewarding stimuli, or in other words by enhancing BIS over BAS activity. This change in activity between the two postulated motivational brain circuits appeared to become part of the daily behavioral organization in our mouse model.

Anhedonia is a prominent behavioral feature of our 'rat stress' model, that is manifested as a consequence of the presumed reduced activity of the mesolimbic dopaminergic circuit. In order to test for anhedonia our studies were performed along the conceptual framework developed by Huston and Oitzl (Huston and Oitzl 1989) aimed to integrate naturally occurring reinforcers as memory promoting rewards. Here we report that access to sugar as post-training reward in close context with learning indeed partially restored the spatial memory impairment of the chronically stressed mice. This discovery is a further demonstration of the *predictive* validity of our chronic stress model.

Our findings raise the following questions for further study:

(i) ***Translational perspective***. Since the negative inhibitory bias of stressed mice was partially counterbalanced by exposure to a sugar reward, it is tempting to translate this finding to a therapeutical perspective in the human. Environmental enrichment of the home situation has been shown to increase the well-being of both humans and mice by lowering anxiety levels with a positive influence on learning and memory performance (Walker and Mason 2011). Alternatively, the combination of extinction training in a fear conditioning paradigm with anti-depressant treatment was recently shown to remodel the memory circuit through local neurotrophic activity (Karpova et al. 2011).

These two examples demonstrate that external cues can help (at least partially) to overcome the effects induced by previous stressor for better or worse. It would be of interest to study whether exposure to positive stimuli can also be used as 'animal therapy', translating human findings back to the design of the experimental procedures and measurements. This implies that positive stimuli during behavioral testing may increase the well-being of the animals. Alternatively, in translational perspective, the combination of this type of psychotherapy based on reward may help the efficacy of anti-depressants in remodeling neural circuitry underlying the BIS/BAS reciprocity. In further experiments the actual measurement of BIS/BAS activity, obviously, is required to substantiate this notion.

(ii) ***Dopamine and opioid signaling in the reward circuitry.*** A diminished functioning of the reward mechanism is evident in patients suffering from depression, especially in the mesolimbic dopamine system (Nestler and Carlezon 2006). In our chronic stress model, post-training reward modulated memory performance, and sucrose preference eventually led to an increase in sucrose consumption over time. To account for this phenomenon Treadway and Zald (Treadway and Zald 2011) differentiated between

consumatory (hedonic – 'liking') and motivational ('wanting') aspects of reward. Whereas the mesolimbic system is involved in motivational processes, the opioid system underlies the sensation of the pleasure obtained from drinking a sweet solution or disgust when something is bitter. To better understand this dissociation we would need to examine the impact of chronic stress in our animal model of depression on dopamine and opioid signaling.

(iii) ***HPA reactivity.*** More data needs to be collected on the HPA reactivity to support the *face* validity of the neuroendocrine system in our 'rat stress' model of depression. This refers to the neuroendocrine characterization of the 'rat stress' model. Also, the improved performance in stressed mice by post-training sugar might result from the attenuation of HPA axis activity following this reward. It would be of interest to examine if CORT dynamics acquires the optimal range required for promotion of learning and memory processes.

(iv) ***The "antistress" drug mifepristone.*** The daily recurrent blockade of the GR with the very high doses of mifepristone triggered in the mouse a rebound surge of endogenous CORT that subsequently mediated a negative feedback action and progressively downregulated HPA axis activity. This paradoxical effect of mifepristone in the mouse occured in the face of a persistent limbic MR activation, which incidentally also explained the concomitant change in explorative behavior towards a serial search strategy. The finding points to the mechanism underlying the beneficial effect of the "anti-stress'' drug mifepristone for stress-related mood disorders like depression.

The above mentioned conclusion is based on findings in the naïve unstressed mouse and needs to be extrapolated to the chronic stress model. If this extrapolation holds, it provides a criterium for *predictive* validity that explains how 'normalization' of aberrant circadian cortisol secretion may occur in patients suffering from psychotic major depression treated daily with very high doses of mifepristone (Belanoff et al. 2001a; Thomson and Craighead 2008). However, the kinetics of mifepristone is very different in mouse and men, because in human the antiglucocorticoid is protected against rapid metabolism and clearance by binding to a circulating α_1-glycoprotein lacking in the mouse. It seems that the dose and mode of administration of the GR antagonist is essential for downregulation of HPA axis activity and therapeutic efficacy, but this needs to be further examined.

(v) ***MR:GR balance.*** The paradoxical strengthening of negative feedback inhibition of CORT secretion by recurrent administration of mifepristone is most likely achieved by integration of MR- and GR-mediated effects. Hence, besides anti glucocorticoid modulation of the MR:GR balance also enhancing the MR- mediated actions in the brain may provide an interesting alternative lead towards a novel class of antidepressants and/or antipsychotics. That one particular gene variant (haplotype) of the MR is associated with optimism therefore provides a highly exciting novel lead (Klok et al. 2011).

Chapter 10

Summary

Worldwide, depression is among the leading causes of disability. It is a mood disorder that leads to substantial impairments in an individual's ability and pleasure to take care of everyday responsibilities. Antidepressant medications and brief structured forms of psychotherapy are still ineffective for 20-40% of the affected individuals. Therefore, more effective fast acting medicines are therefore urgently needed. What hampers the progress in new drug discovery is the complex nature of depression which involves multiple brain processes. A promising perspective for new drug targets is that the etiology of depression has been linked to the inability of the affected individual to cope with chronic stress. The aim of the research described in this thesis was to develop an animal model which would express a wide range of emotional, behavioral and neuroendocrine signs and symptoms of depression, based on exposure of mice to a chronic stressor. Using this model, new drug targets could be revealed and current pharmacologic treatment tested on a wide range of processes.

The glucocorticoids cortisol and corticosterone (collectively called "CORT") are secreted by the adrenal glands after activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis in response to stress. This response occurs on top of its ultradian (hourly burst in secretion) and circadian (24h) rhythms. CORT and the other hormones of the HPA axis are powerful neuro-endocrine mediators of stressful environmental stimuli. They coordinate adaptive functions of brain and body via the mineralocorticoid- and glucocorticoid receptor (MR and GR).

Using our chronic stress mouse model we investigated how adaptation to stress can become impaired and how this impaired adaptation is capable to precipitate emotional and cognitive disturbances as characteristic features of depression. In this line of reasoning, chronic stress leads to an altered pattern of HPA axis activity which is considered causal to the pathogenesis of depression. The key symptom of depression studied in this thesis is *anhedonia*, which is defined as a decrease in the sensitivity for a reward, i.e. positive stimuli.

We have tested the hypothesis that chronic stress alters glucocorticoid signaling thereby disturbing the appraisal processes that underlie the expression of anhedonia. We monitored the expression of reduced responsiveness to positive stimuli by assessing learning and memory performance, emotionality and endocrine response patterns during and after cessation of the chronic stress.

Before we assessed the effects of chronic stress on psychoneuroendocrine parameters in our mouse model, we characterized the basal 24h circadian activity patterns of selected

HPA axis markers in male 3, 9, and 16 months old C57BL/6J mice. In **Chapter 2** the results were described for the 9 and 16 month old mice in comparison to 3 month old mice.

Whereas 9 month old mice expressed a relative hypercorticism (high CORT level), 16 month old mice displayed a relative hypocorticism (low CORT level). Mineralocorticoid- (MR) and glucocorticoid receptor (GR) mRNA expression in the hippocampus were significantly decreased in 9 month old mice, whereas in 16 month old mice, the expression of both MR and GR was similar to that observed in young animals. The parvocellular hypothalamic paraventricular nucleus (PVN) expressed very high vasopressin mRNA in 16 month old mice, which was subject to circadian variation in 3 and 9 months old mice.

In conclusion, basal 24h-circadian HPA axis activity and expression of some of its central regulatory markers are age-dependent in mice. It is showing an inverted U-shape pattern with highest activity at 9 months of age at least with respect to CORT. For the remainder of the studies we continued using male 3 months old mice as to avoid interference of the basal endogenous and stress induced CORT secretion by age, induced by our chronic stress model.

Conventional drug delivery methods (e.g., subcutaneous, intraperitoneal, *per os*) are intrusive and consequently, can evoke a stress response. This additional stress response can interfere with the pharmacological action of the drug and behavior being studied. Because we wanted to study the effect of GR antagonist RU38486 (i.e. mifepristone – MIF) in naïve and stressed mice, we devised a novel non-invasive, stress-free method of drug delivery via oats in mice in, as described in **Chapter 3**. We measured CORT in blood plasma in response to conventional drug delivery methods and following drug delivery via oats.

Oat consumption induced a small increase in CORT concentrations after 15 min (< 50ng/ml) that returned to the initial low resting levels after 30 min (< 10ng/ml). Gavage and intraperitoneal vehicle injections resulted in long-lasting CORT elevations (> 100ng/ml and ~ 50ng/ml after 30 min and at 60 min respectively). To determine whether it would be possible to produce a pulse with exogenous CORT, three different CORT doses were added to the oats. These doses were offered to adrenalectomized mice as to eliminate the contribution of endogenous CORT. Adding CORT to oats resulted in a 3-fold higher plasma CORT concentration in the 15.0mg/kg-group (± 250ng/ml) compared to the 4.5mg/kg-group at t=30 and t=90 min. Interestingly, the administration of mifepristone (MIF -200mg/kg) via oats elevated plasma CORT for at least eight hours in non-stressed mice.

Concluding, oat delivery is a good, practical and useful non-invasive method for the delivery of glucocorticoid ligands. The method of administration induces a very low stress-CORT level, and allows also to mimic a CORT pulse. This method was applied in **Chapter 4**.

In **Chapter 4** the effects of single and repeated GR blockade using MIF on circadian CORT patterns and stress-induced neuroendocrine and behavioral responses were described. We designed a study to mimic the protocol which has proven successful in the treatment of patients that suffer from psychotic major depression. Naïve male C57BL/6J mice were offered MIF (200mg/kg) *per os* by oats, either once (1xMIF) or once per day on 7 consecutive days (7xMIF) or vehicle (VEH).

Whereas single administration of this very high dose of the GR antagonist resulted in very high CORT concentrations, repeated GR antagonism progressively downregulated HPA axis activity towards a normal CORT output. To explain this unexpected phenomenon we reasoned that in fact the very high CORT level remained elevated beyond the actual presence of the GR blockade, and hence were capable to exert a strong feedback signal suppressing HPA axis activity. This GR-mediated CORT feedback signal persisted because of its genomic nature, long after return of CORT to even below baseline levels as observed at 32h after the first administration. However, 24h after MIF administration, the mice were still capable to show a rapid stress-induced increase in CORT following exposure for 5 min to the circular hole board. After 7 cycles of MIF, the CORT feedback has proceeded to such an extent that neither MIF nor stress is capable to activate the HPA axis.

The contribution of the brain CORT receptors was also determined. While brain and pituitary GR were subsequently blocked by MIF and activated by endogenous CORT over several circadian cycles, the brain MR is freely accessible by circulating CORT under any condition. In response, the hippocampal MR expression was initially lower during high levels of CORT, but increased upon repeated GR antagonist exposure. Particularly within the hippocampal CA2 region at the time CORT exposure was back to baseline. The patterns of MR and GR activation during the course of daily repeated GR antagonism was also expressed in the choice of search strategy employed. Whereas 1xMIF mice were hyperactive, the 7xMIF mice showed relatively more serial search patterns than 1xMIF and VEH treated animals. This suggests an increased role of MR-mediated limbic function.

In conclusion, our data revealed that the recurrent daily blockade of GR by the very high dose of MIF did not produce the expected lasting hypercorticism. Instead,

it led to downregulation of basal and stress-induced HPA axis activity. Possibly this downregulation is caused in part by the long lasting CORT feedback activity hat becomes prevalent during timepoints when the GR antagonist dissociates from GR. This recurrent blockade and activation of the GR is thought to proceed in cooperation with a limbic MR mediated mechanism that may account for the reported (Wulsin et al. 2010) MIF-induced suppression of excitatory, and enhancement of inhibitory inputs to the HPA axis.

To determine which behavioral task would be most suitable to study a variety of behavioral responses that are indicative for chronic stress-induced improvement or impairment of learning and memory processes (see **Chapter 6, 7** and **8**), we compared two commonly used behavioral paradigms for (non-) spatial learning and memory: the circular hole board and the water maze. Additionally, we studied the modulation of spatial memory by reward as a post-training positive reinforcer as described in **Chapter 5**. Free access to sugar was chosen as a post-training reinforcer and was provided immediately (0h-sugar) after training, or with a delay of 4h (4h-sugar), while the controls did not receive sugar.

In both tasks, '0h-sugar mice' showed superior performance as indicated by shorter latencies and distances to the trained spatial location. The memory facilitating effect of sugar became detectable at distinct times during training: on the circular hole board from the first trial onwards, whilst in the water maze on training days 4 and 5. Both the '0h and 4h-sugar'-rewarded mice kept their superior performance during the free exploration/swim trial as expressed by their more persistent search strategies for locating the exit hole or platform. We showed that a sugar reward given immediately after the training trials each day (0h-sugar) reinforced memory processes via enhancement of consolidation.

These findings support the integrative theory of reinforcement and memory advanced by Huston & Oitzl (1989). This is in particular the case for the circular hole board procedure, which provides a broader range of behavioral responses that can be studied. The experimental set-up of the circular hole board allows differentiation of learning and memory processes as well as detection of alterations in reward processes. Accordingly, we have used the circular hole board in the experiments described in **Chapters 6, 7** and **8** designed to determine the consequences of the sucrose award for behavioral performance tested our animal model of depression.

The effects of chronic stress studied in a variety of animal models are mainly assessed in short-lasting test-situations that have task-inherent features of novelty. Sometimes these situations even include exposure to physical stressors. In **Chapter 6** we reported the

impact of chronic stress on the daily organization of behavior in the familiar environment of the home cage, during and after cessation of the stressor in our animal model (i.e., the repeated and unpredictable exposure of mice to rats without physical contact). In addition, exploration of a novel environment was determined.

Continuous longitudinal observation revealed that 'rat stress' decreased exploratory and foraging activity as characterized by increased time spent in the shelter and less time spent in the open area. The brain reward mechanism was affected as indicated by reduced sucrose consumption and inhibition in sucrose preference development. Stressed mice used a more perservative strategy during exploration of a novel environment, whilst general locomotor activity was unaffected. Interestingly, already the control procedure, that includes spending the same amount of time in another cage without rat exposure, disrupted the organization of behavioral activity patterns, albeit to a lesser degree. In some aspects this was different than observed in rat-stressed mice.

The results support our notion that mice repeatedly exposed to rats might serve as a model of (human) chronic stress. Distinct behavioral changes in explorative and foraging activities, as well as the reduced response to a rewarding stimulus, suggest that negative changes in the reward system have occurred during chronic 'rat stress', in the context of changes in circadian CORT secretion. The loss of interest in pleasurable activities is known as anhedonia which is a hallmark, not only in individuals suffering from chronic stress exposure, but also of depression.

In **Chapter 7** we reported a combination of methodologies as described in the previous chapters, to determine whether the expression of anhedonia in our 'rat-stress' paradigm would be measurable using additional read-out parameters. Following cessation of the chronic stressor we assessed: learning and memory performance, facilitation of memory by reward, reward sensitivity, the emotional response and CORT levels.

It appeared that chronic 'rat stress' induced alterations in three domains of reward processing as indicated by (1) suppression of behavioral reactivity to novelty; (2) enhanced memory processes in response to sugar reward: spatial performance improved in control mice, whereas sugar reward "ameliorated" the impaired performance of stressed mice to the level of non-stressed controls without sugar; (3) increased sucrose and water intake: stressed mice that had received sugar post-training preferred to drink water at the location of prior sucrose consumption. Finally, the total CORT secretion during the light period of the day increased from day 1 to day 7, following the first week after 'rat stress'.

Taken together, chronic 'rat stress' altered the circadian CORT secretion pattern over time, impaired spatial memory and increased caloric intake. These alterations show, in addition to the previously observed diminished response to positive stimuli, that mice exposed to our chronic stress paradigm express anhedonia, which is supported by the changes in the three read-out parameters used. Sugar offered in the context of spatial learning partially rescued stress-induced, emotional and cognitive impairments. Collectively, these findings suggest that reward can ameliorate part of the negative consequences of chronic stress on memory processes.

Acute stress has been shown to modulate different memory systems to guide behavior in favor of caudate nucleus-dependent stimulus-response learning and memory at the expense of hippocampus-dependent spatial learning. In **Chapter 8,** a translational study was described where we examined in mice and humans, whether chronic stress has similar consequences as acute stress for the use of either one or both memory systems.

In our animal test, male C57BL/6J mice exposed to chronic 'rat stress' more often used a stimulus-response strategy than control mice for locating the exit hole on the circular hole board task. Thirty three percent of the stressed mice altered their strategy to stimulus-response or habit learning, while none of the control mice did; the controls all adhered to the spatial strategy. In the human test, forty healthy young men and women were divided into a "high chronic stress" and a "low chronic stress" group based on their answers posed in a questionnaire (the "Trier Inventory of Chronic Stress"-TICS) to identify symptoms of chronic stress. The subjects were trained in a 2D task where they had to remember the location of an object. We found that 94% of the participants of the "high chronic stress" group more often used the stimulus-response strategy, while this was the case only for 52% of the "low chronic stress" participants.

Chronic stress seemed to affect the quality of learning. This means that chronic stress affects *which* memory system is involved in the process of learning and *how* an individual learns. The induced shift towards a more rigid habit of stimulus-response learning strategy appears to be one of the consequences of chronic stress that can make an individual more vulnerable to the negative consequences, when exposed to additional stressors in the future.

As discussed in **Chapter 9** the following conclusions were reached

1) Our chronic stress model of *repeated and unpredictable* exposure of mice to rats proves to fulfill criteria of construct and face validity for depression.

2) The long lasting decrease in responsiveness to positive stimuli, which is considered indicative of anhedonia, served as presumed symptom of depression in our chronic stress model.

3) A history of chronic stress produces in both mice and men a shift towards a more rigid habit of stimulus-response learning.

4) Rigidity i.e., behavioral inhibition and habit learning, appears to be one of the consequences of chronic stress that can make an individual more vulnerable to the negative consequences of subsequent periods of stress.

5) The new methodology to reduce stress by either administration of the "anti-stress'' drug mifepristone or by providing positive and rewarding stimuli during behavioral testing, increases the well-being of the animals and may - in translational perspective - protect against depression.

6) The daily recurrent blockade of the GR with a very high dose of mifepristone may downregulate HPA axis activity because of the rebound surge of endogenous CORT. This subsequently mediates a negative feedback action in the face of a persistent limbic MR activation with concomitant changes in explorative behavior

7) Modification of the MR-GR balance may provide an interesting lead towards a novel class of antidepressants and/or antipsychotics.

Hoofdstuk 10

Samenvatting

Wereldwijd behoort depressie tot een van de meest voorkomende ziektebeelden. Het is een stemmingsstoornis welke zich kenmerkt door een verminderde levenslust of zware neerslachtigheid, en die ertoe bijdraagt dat een individu moeite heeft met het uitvoeren van alledaagse taken. Behandeling met antidepressiva en kortdurende, gestructureerde psychotherapie hebben geen effect bij 20-40% van de mensen. Effectievere geneesmiddelen en behandelingen zijn daarom dringend nodig.

De complexe aard van depressie, waarbij meerdere hersenfuncties betrokken zijn, bemoeilijkt de ontwikkeling van nieuwe geneesmiddelen. Er zijn aanwijzingen dat chronische stress leidt tot verhoogde kans op ontwikkeling van depressie, in individuen met een genetische aanleg. Stress leidt tot activatie van de Hypothalamus-Hypofyse-Bijnier- (HHB-) as, waarna de bijnieren de glucocorticoïden (stresshormonen) meer cortisol en corticosteron (verder aangeduid als “CORT”) in het bloed uitscheiden. Deze verhoging in de CORT concentratie komt bovenop de reeds aanwezige ultradiane en circadiane ritmes van het stresshormoon. Het ultradiane ritme geeft de amplitude en frequentie van de pulsatiele hormoonafgifte aan Het circadiane ritme weerspeigelt de schommelingen in het dag-en nachtritme. Bij het aanbreken van de actieve periode (bij mensen is dit het begin van de lichtperiode; bij muizen is dit het begin van de donkerperiode) bereikt het circulerend CORT niveau zijn circadiane piek waarde. Deze wordt gekenmerkt door een grotere amplitude in de pulsen van CORT afgifte door de bijnieren die ieder uur voorkomen. De effecten van CORT komen tot stand na binding aan de mineralocorticoid- en glucocorticoid receptoren (MR en GR respectievelijk). MR is betrokken bij de activatie van de HHB-as in reactie op een stress, en bij de gedragsresponse dien ten gevolge. Wanneer door de stressde CORT concentratie toeneemt, wordt de GR geactiveerdt dat leidt tot normalisatie van de HHB-as activiteit. Tevens is de GR betrokken bij leer- en geheugen processen. Deze endocriene reactie op stress zorgt ervoor dat (i) het individu zich kan aanpassen aan de stressvolle situatie en (ii) het individu leert hoe er mee om te gaan indien een soortgelijke stressvolle situatie zich weer voordoet.

Het doel van het in dit proefschrift beschreven promotie-onderzoek was om een muismodel voor depressie te ontwikkelen. Het model is gebaseerd op de blootstelling van muizen aan de aanwezigheid van ratten, wat diende als een chronische stressor.

Gebruikmakend van ons muismodel is onderzocht hoe chronisch stress kan leiden tot verstoringen in emotionele en cognitieve processen die kenmerkend zijn voor symptomen van depressie zoals die mensen voorkomt. Het meest kenmerkende symptoom van depressie is een verminderde reactie op positieve prikkels, dat *anhedonie* wordt genoemd. *Anhedonie* is in ons muismodel gemeten door te bepalen of er een

afname of verandering plaatsvond in de reactie op een aangeboden positieve prikkel (beloning).

Alvorens we de effecten van chronische stress op HHB-as activatie en activiteit in ons muismodel hebben bestudeerd, werd eerst de basale circadiane (licht/donker ritme) 24 uurs CORT concentratie bepaald. Tevens is op de tijdstippen dat de CORT concentratie het laagst en het hoogst is, de expressie van MR en GR in de hersenen, en het adrenocorticotroop hormoon (ACTH) bepaald in 3, 9, en 16 maanden oude mannelijke C57BL/6J muizen. Deze resultaten staan beschreven in **Hoofdstuk 2**.

De 9 maanden oude muizen vertoonden *hyper*corticisme (te veel CORT). Daarentegen, de 16 maanden oude muizen vertoonden *hypo*corticisme (te weinig CORT), also ook een verhoogde ACTH concentratie in het bloed gedurende de dag. De expressie van MR en GR mRNA was lager in de hippocampus van de 9 maanden oude muizen, terwijl de expressie in de 16 maanden oude muizen vergelijkbaar was met die van 3 maanden oude muizen. Verder liet de parvocellular paraventriculaire kern (PVN) in de hypothalamus van de 16 maanden oude muizen een zeer hoge expressie van vasopressine mRNA en een significante verhoging van MR mRNA zien.

We concluderen dat de activiteit van de HHB-as mede door de leeftijd wordt bepaald en dat de maximale CORT afgifte over de leeftijd zich laat zien als een omgekeerde U-vorm. Niet de oudste dieren, maar de 9 maanden oude dieren vertonen de hoogste maximale CORT afgifte. De 3 maanden oude muizen hebben een HHB-as activiteit waarbij we voorzagen dat de effecten van chronische stress het best meetbaar zou zijn. Voor de rest van de in deze thesis beschreven onderzoeken is gebruikt gemaakt van de 3 maanden oude muizen.

De traditionele methoden voor het toedienen van geneesmiddelen bij muizen (b.v. subcutaan*,* intraperitoniaal of *per os*) zorgen voor een stressreactie. Deze stressreactie kan ertoe leiden dat zowel de werking van het geneesmiddel wordt beinvloed, als ook het eventuele daarna bepaalde gedrag. Daar we in ons chronisch stressmodel de GR antagonist RU38486 (ook wel bekend als mifepristone –MIF- dat klinische resultaten laat zien) wilde gaan toedienen (zie **Hoofdstuk 4**), en we dat gepaard wilde gaan met een zeer lage stressreactie, is een nieuwe niet-invasieve en stressvrije methode ontwikkelt welke beschreven staat in **Hoofdstuk 3.** Voor deze toediening is gebruikt gemaakt van havervlokken waarop de te in te brengen stof is toegevoegd. Deze toediening via havervlokken werd vergeleken met toediening via injecties en orale toediening, door de stress/CORT reactie in het bloedplasma te meten.

De consumptie van de onbehandelde havervlokken veroorzaakte een kleine toename in de CORT concentratie na 15 minuten (< 50ng/ml), welke na 30 minuten terugkeerde naar de eerdere lage basale waarde (< 10ng/ml). Zowel de toediening *per os* als de intraperitoneale injectie resulteerde in een langdurige CORT verhoging 30 en 60 minuten na toediening (respectievelijk > 100ng/ml en ~ 50ng/ml).

Om te weten of de havervlok methode ook gebruikt kon worden om CORT-pulsen te induceren, werden 3 verschillende doseringen van CORT toegediend. Dti gebeurde bij muizen waarbij bijnieren waren verwijderd, zodoende dat het endogene CORT niet van invloed was op de CORT metingen. Het toedienen van CORT via havervlokken resulteerde in een 3 maal hogere plasma-CORT waarde in de 15.0mg/kg groep (± 250ng/ml) in vergelijking met de 4.5mg/kg groep 30 en 90 minuten later. De toediening van mifepristone (MIF - 200mg/kg) via havervlokken leidde eveneens tot een verhoging van plasma CORT die ten minste acht uur duurde.

Het toedienen van medicatie via de consumptie van havervlokken is een goede, niet-invasieve methode. De toediening gaat gepaard met een zeer lage stress/CORT reactie en met kan er tevens een CORT-pulse mee generen. Deze toedieningsmethode wordt verder toegepast in **Hoofdstuk 4**.

In **Hoofdstuk 4** zijn de effecten beschreven van een éénmalige en herhaalde GR blokkade middels de GR-antagonist MIF, op circadiane CORT veranderingen en stress-geïnduceerde neuro-endocriene en gedragsmatige reacties. De opzet van dit experiment kwam wat betreft de dosis en de tijdsduur van behandeling overeen met de succesvol gebleken therapie toegepast bij patiënten die lijden aan een psychotische depressie. Mannelijke C57BL/6J muizen gedurende 1 week/1x per dag een zeer hoge dosis MIF (200mg/kg) via havervlokken toegediend (zie **Hoofdstuk 3**). De CORT concentratie in bloed werd gedurende 24 uur gemeten op dag 1 (1XMIF) en dag 7 (7xMIF) na toediening, en vergeleken met een controle groep, die havervlokken kregen met daarin alleen het oplosmiddel.

De éénmalige toediening van de zeer hoge dosis van MIF resulteerde in zeer hoge CORT concentraties, terwijl herhaalde toediening van dezelfde hoge concentratie (7xMIF) leidde tot een geleidelijke verlaging in HHB-as activatie. Een mogelijke verklaring voor dit onverwachte resultaat is dat de hoge CORT concentratie de activiteit van de HHB-as onderdrukt via GR activatie, nadat MIF van de GR gedissocieerd is. Deze terugkoppeling via CORT bleef tot 32 uur na de laatste toediening van MIF aanwezig vanwege genomische processen, waarbij de CORT concentratie zelfs nog lager was dan eerder gemeten.

De expressie van de CORT receptoren MR en GR in de hersenen zijn ook bepaald. Terwijl de GR in de hersenen en hypofyse achtereenvolgens door MIF werden geblokkeerd, en door het endogene CORT werden geactiveerd, zijn de MR in de hersenen vrij toegankelijk voor CORT. Als gevolg hiervan was de MR expressie in de hippocampus aanvankelijk lager tijdens de hoge CORT concentratie, maar was deze verhoogd na herhaalde toediening van de GR-antagonist in het CA2 gebied van de hippocampus. Toediening van MIF leidde ook tot een verandering in de gedragsparameters gemeten in de 'circular hole board test' (= een ronde plaat met gaten, zonder wanden, waar de dieren getest worden op exploratief gedrag). De 1xMIF muizen waren hyperactief, terwijl de 7xMIF muizen een voorkeur hadden voor het gebruik van een meer seriële zoekstrategie. Een dergelijke voorkeur voor zoekstrategie wijst eveneens op beïnvloeding van de centrale MR functie die betrokken is bij de beoordeling van een situatie, en bij de vervolgens gekozen strategie om met die situatie om te gaan.

Concluderend, dagelijkse herhaalde toediening van een hoge dosis MIF, leidde in tegenstelling tot onze verwachting niet tot hypercorticisme, maar juist normalisering van circulerende CORT gehaltes opleverde. De onderdrukking van de HHB-as activiteit komt vermoedelijk tot stand doordat na het dissociëren van MIF van de GR, de hoge CORT concentratie de GR activeert en zo de HHB-as activiteit onderdrukt. De dagelijkse MIF toediening gaat gepaard met MR gerelateerde processen, omdat deze receptor door de antagonist ongemoeid gelaten wordt (Wulsin et al., 2010). De activatie van MR kan ook de activatie van de HHB-as remmen.

Voordat de effecten van chronisch stress op leer en geheugenprocessen werd bepaald (zie **Hoofdstukken 6, 7,** en **8**), is bepaald welke gedragstaak daarvoor het meest geschikt zou zijn. Hiervoor zijn twee veel gebruikte gedragstaken vergeleken: de 'circular hole board' en 'de water maze', zoals beschreven in **Hoofdstuk 5**. Tevens werd de beïnvloeding van het ruimtelijk geheugen door beloning in deze gedragstaken onderzocht. Als beloning was gekozen het vrijwillige innemen van een hoeveelheid suikerkorrels in de thuiskooi, direct (0-uur groep) of 4-uur (4-uur groep) na de leertaak. De controle dieren kregen geen suiker.

Op zowel de 'circular hole board' als in de 'water maze' vertoonden de muizen die direct na de training suiker als beloning kregen (0-uur) superieure prestaties. Ze vonden de ontsnappingslocatie sneller en onthielden die beter. Het effect van de suikerbeloning op de leerprestatie was zichtbaar op verschillende tijdstippen tijdens de training. Bij de 'circular hole board' was dit effect reeds meteen zichtbaar vanaf de eerste trainingsdag, maar bij de 'water maze pas tijdens de 4de en 5de trainingsdag.

De resultaten tonen aan dat een suikerbeloning die direct na de training gegeven wordt, het geheugen van het zojuist geleerde gedrag kan versterken. Deze bevinding is in overeenstemming met de theorie 'reinforcement and memory' opgesteld door Huston & Oitzl (1989). Op basis van de bevindingen in **Hoofdstuk 5** is gekozen voor de 'circular hole board' in de vervolgexperimenten om de rol van beloning en de effecten van chronische stress op leer- en geheugenprocessen (**Hoofdstukken 6, 7** en **8**) te bestuderen

In **Hoofdstuk 6** werd het effect van chronische stress op de dagelijkse organisatie van foeragerend en exploratief gedrag in de bekende omgeving van de thuiskooi onderzocht. Als stressor werd gekozen voor de herhaalde blootstelling van de muis aan de aanwezigheid van een rat. De rat kon door de muis wel worden geroken en gezien, maar zonder dat er fysiek contact mogelijk was.

Deze 'rat stress' leidde tot een verlaging in verkennende en foeragerende activiteiten (regelmatig terugkerend zoeken en vinden van voedsel) van de muis in de thuiskooi. Er werd meer tijd in de schuilplaats doorgebracht en minder tijd in de open ruimte, wat duidt op angst. Tevens vertoonden de gestresste muizen een verminderde consumptie van sucrose en een langzamere ontwikkeling in de voorkeur voor een sucrose oplossing boven water. Daarbij gebruikten de muizen een meer persistente zoekstrategie tijdens het verkennen van de 'circular hole board' terwijl de locomotorische activiteit onveranderd bleef.

De resultaten laten zien dat ons chronisch stress model, d.i. de herhaalde blootstelling van muizen aan de aanwezigheid van een rat ('rat stress'), een verandering in het beloningssysteem teweegbrengt. Dit is af te leiden uit een afname in verkennende en foerageeractiviteiten, als ook een verminderde reactie op een belonende stimulus. Deze verminderde reactie op een beloning kan gedefinieerd worden als *anhedonie*, een belangrijk symptoom dat kenmerkend is voor depressie.

In **Hoofdstuk 7** werd een combinatie van eerder toegepaste en ontwikkelde methoden gebruikt om verdere effecten van de 'rat stress' procedure op leer-, geheugen- en beloningsprocessen te bepalen. Dit gebeurde in een nieuwe omgeving buiten de thuiskooi. Hiertoe werden de volgende parameters bepaald na afloop aan de blootstelling aan de chronische stressor: leer- en geheugenprocessen met en zonder suikerbeloning, de reactie op positieve prikkels en de CORT concentratie in het bloed.

De chronische 'rat stress' onderdrukte de gedragsrespons tijdens blootstelling aan een nieuwe omgeving en verminderde het effect van suikerbeloning op de geheugenprocessen. Suikerbeloning verbeterde de ruimtelijke leerprestatie bij controle

muizen. Daarnaast werd de vermindere leerprestatie bij gestresste muizen verbetert door de beloning, waardoor ze evengoed presteerden als de controle muizen die geen suikerbeloning hadden gekregen. Verder vertoonden de gestresste muizen die na training op de 'circular hole board' de suikerbeloning hadden kregen, een voorkeur voor de fles met water die was aangeboden op de plaats waar eerder een fles met sucrose was geplaatst. Een dag na afloop van de chronische 'rat stress' periode was de totale CORT secretie tijdens de lichtperiode hoger dan die een week later gemeten.

Concluderend, de chronische 'rat stress' zorgt voor veranderingen die wijzen op verschijnselen van anhedonie. Daarnaast blijkt een suikerbeloning de door stress veroorzaakte emotionele en cognitieve stoornissen gedeeltelijk te herstellen. De resultaten suggereren dat een beloning een deel van de negatieve gevolgen van de chronische 'rat stress' op het geheugenproces, kan verbeteren.

Informatie wordt door de hersenen op verschillende manieren verwerkt en bewaard voor gebruik indien nodig. Eerdere studies hebben aangetoond dat door acute stress eerder gebruik gemaakt wordt van een directe stimulus-respons strategie dan van een hippocampus-afhankelijke ruimtelijke leer- en geheugenprocessen om een leertaak uit te voeren. In **Hoofdstuk 8** is bij muizen en mensen onderzocht of chronische stress hetzelfde effect heeft als acute stress, op het gebruik van één of beide leer en geheugensystemen in een vergelijkbare leertaak. Hiertoe moesten de muizen de locatie van een uitgang vinden op de 'circular hole board', terwijl de mensen de locatie van een voorwerp dienden te onthouden in een 2-dimensionele leertaak.

De mannelijke C57BL/6J muizen die blootgesteld waren aan 'rat stress' maakten vaker gebruik van een stimulus-respons strategie (33%) dan de controle dieren, die vasthielden aan de hippocampus-afhankelijke zoekstrategie. Veertig gezonde proefpersonen werden verdeeld in een 'hoge chronische stress' en een 'lage chronische stress'-groep op basis van hun antwoorden op vragen uit een vragenlijst (de "Trier Inventaris van Chronische Stress"-TICS), waarmee de symptomen van chronische stress bepaald kunnen worden. Van de proefpersonen behorende bij de "hoge chronische stress" groep gebruikte 94% vaker de stimulus-respons leerstrategie, terwijl dit bij de"lage chronische stress" groep slechts 52% was.

Concluderend, chronische stress beïnvloedt voornamelijk de kwaliteit van het leren. Dit betekent dat chronische stress beinvloedt *welk* leer- en geheugensysteem wordt gebruikt, en zo dus *hoe* een individu leert. De verschuiving naar het gebruik van de meer *rigide* vorm van stimulus-respons leren lijkt één van de gevolgen van chronische

stress. Dit kan ertoe bijdragen dat een individu kwetsbaarder wordt voor de gevolgen van een toekomstige stresssituatie.

Zoals beschreven in **Hoofdstuk 9**, komen we tot de volgende conclusies:

1) Ons chronische 'rat stress' model waarbij muizen herhaaldelijk en op onvoorspelbare tijden worden blootgesteld aan de aanwezigheid van een rat, voldoet aan de 'construct en face validity criteria' voor depressie.

2) De langdurig veminderde reactie op beloning, die wordt gezien als kenmerk van anhedonie, werd geinduceerd in ons chronisch stress model als een symptoom van depressie.

3) Een voorgeschiedenis van chronische stress resulteert bij muizen en mensen in een toename van het gebruik van stimulus-response (minder flexibel) leren en gaat ten kosten van het ruimtelijk (meer flexibel) leren.

4) Starheid in gedrag, uitgedrukt als een geremdheid en een minder flexibele manier van leren, lijkt één van de gevolgen van chronische stress die er toe kan leiden dat een individu gevoelig wordt voor toekomstige stressvolle situaties.

5) De nieuwe methodiek om stress (en de gevolgen ervan) te verlagen via toediening van het 'anti-stress' medicijn mifepristone, of via extra positieve prikkels en beloning tijdens gedragstaken vergroot het welzijn van de muizen en kan – vertaald naar de menselijke situatie - beschermen tegen de gevolgen van depressie.

6) De dagelijkse blokkade van GR met de zeer hoge doses van mifepristone kan de HHB-as onderdrukken doordat de verhoging van het endogene CORT de negatieve terugkoppeling van de HHB-as tot stand brengt. Hierbij zijn zowel de GR betrokken die beschikbaar worden na het verdwijnen van de antagonist, als ook de MR die door verhoging in CORT extra geactiveerd worden. De veranderingen in neuroendocriene functie wordt ook gezien in exploratief gedrag dat overgaat van hyperactiviteit naar een meer seriële zoekstrategie bij herhaalde toediening.

7) Een geïnduceerde verandering in de expressie en activatie van de MR-GR kan leiden tot een nieuwe klasse van medicijnen tegen depressie of psychotische aandoeningen.

Hoofdstuk 11

List of publications
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- Behavioral Genetics seminar, March 2007, Wageningen, Netherlands.
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Curriculum Vitae

Sergiu Dalm werd geboren op 09 Augustus 1973 te Delft, Nederland. In 1992 behaalde hij zijn HAVO diploma aan het Maascollege in Maassluis. Aansluitend zette hij zijn studie voort aan de Hoge School voor Laboratorium Onderwijs te Delft, waar hij zijn artikel 12 certificaat verwierf. De bijbehorende 9-maanden stage werd uitgevoerd in het kader van een samenwerkingsproject tussen TNO Preventie en Gezondheid (Prof. Dr. L. Havekes, Dr. M. Mulder), en de afdeling Medische Farmacologie (LACDR / LUMC, Universiteit van Leiden; Prof. Dr. E.R. de Kloet, Prof. Dr. M. S. Oitzl, Dr. J. Grootendorst), gerelateerd aan het thema 'Apolipoproteine-E, Alzheimer en Cognitie'. Hij is met succes in 1997 afgestudeerd. Vervolgens werkte hij als research technician bij de afdeling Medische Farmacologie aan het project 'Stress effects on cognitive performance of apolipoprotein E-knockout mice'. In September 2002 begon hij bij hetzelfde instituut aan zijn promotieonderzoek waarvan de resultaten staan beschreven in dit proefschrift. Dit onderzoek was onderdeel van het ASPASIA project "Cognition and positive emotions in a dysregulated glucocorticoid system".

In Januari 2008 is Sergiu Dalm in dienst getreden bij Quintiles B.V., een bedrijf dat wereldwijd uiteenlopende klinische onderzoeksdiensten levert voor biotechnische en farmaceutische klanten. Tot eind Augustus 2010 is hij gedetacheerd geweest bij het voormalige Centocor B.V. te Leiden, heden ten dage Janssen Biologics B.V. en onderdeel van Janssen Pharmaceutical companies of Johnson & Johnson, alwaar hij de functie van Trial Document Specialist en Quality Compliance Associate vervulde. Sinds September 2010 is hij werknemer bij Janssen Biologics B.V. als Quality Monitoring and Compliance Associate in samenwerking met Global Clinical Operations.

Dankwoord

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