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Consequences of chronic social stress in mice

Dissertation

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I Introduction

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1.4 Scope of the thesis

1.1 Chronic stress as a risk factor

The daily life of people living in western societies seems to become more and more stressful, which results in a dramatic increase of incidences of stress-associated diseases. Chronic work stress has been described to increase the risk to develop metabolic syndrome (Chandola et al., 2006) and stress is also associated with cardiovascular disease. Strong epidemiological evidence supports a significant role of psychosocial stress in the development of coronary heart disease (Holmes et al., 2006; Brydon et al., 2006; Strike and Steptoe, 2004; Rozanski et al., 2005) and chronic stress largely increases the risk of a fatal stroke in older men (Ohlin et al., 2004). Many psychiatric diseases are also associated with high levels of stress. For example, strong psychosocial stress has been reported to increase the risk to develop Alzheimer's disease a twofold (Wilson et al., 2003). Major depression, which is one of the most common mental disorders worldwide and is predicted by the World Health Organization to be the leading cause of disability by 2020, can be caused or facilitated by chronic stress exposure. Work stress has also been correlated with an increased risk for depression (Wang et al., 2005) and stress has been shown to accelerate disease processes and cause neuronal degeneration (Gold et al., 1988; Nestler et al., 2002; Tafet and Bernardini, 2003). The notion that chronic stress exposure could be causal for the development of affective disorders is supported by findings of altered hypothalamic-pituitary-adrenal axis function (Holsboer, 2000; Ising et al., 2007) and increased corticotropin releasing hormone levels in depressed patients, which is ameliorated by antidepressant treatment (Nemeroff et al., 1984; De Bellis et al., 1993).

However, many authors have also pointed out that stress per se is in most cases not sufficient to cause disease. Not all people become ill after serious, long-lasting stressful experiences. Others develop a disease after having experienced stress which was comparably mild. Stress also constitutes a risk factor for many diverse diseases and every individual seems to be affected in a different way. Kendler and colleagues hypothesized that genetic factors influence the risk of a manifestation of major depression partly by altering the sensitivity of individuals to the depression-inducing effect of stressful life events (Kendler et al., 1995). This emphasizes the urgency to identify genetic or epigenetic factors, which determine the vulnerability of an individual to stressful life events. This issue can only partly be answered by epidemiological studies, as non-experimental studies in human patient populations always face major methodological problems, including the general inaccuracy of life event reporting and dating (Kessler, 1997). It is therefore

inevitable to adjoin data from epidemiological studies with results from several preclinical approaches using animal models.

1.2 Animal models for chronic stress

Animal models for human diseases are rated according to three different criteria: face validity, predictive validity and construct validity (Willner, 1984; Willner and Mitchell, 2002). Face validity means that the phenotype created by the model should be highly similar to the human disease characteristics and replicate a number of the specific symptoms. This criterion can only be accomplished for a subset of disease characteristics, like the neuroendocrine, physiological or behavioural phenotype. Other disease characteristics, like suicidal behaviour of depressed patients, are often impossible to mimic in animals. Predictive validity describes whether drugs, which have been shown to be specific and effective in humans, can pharmacologically manipulate the disease phenotype. This validity criterion is facing the problem, that there may be no specific and effective drugs for the disease available. In the case of antidepressants, all available drugs suffer from a high non-responder rate and a late onset of therapeutic efficacy. Finally, the term construct validity implies that the etiology of the disease in the model is homologous and is based on an empirical and theoretical relationship to the disorder it models. This validity criterion is probably the hardest criterion to fulfil for an animal model, as the "construct" of the disease is often still unclear and the object of investigation. However, if for instance specific risk factors have been identified for a disease, they should be implemented in a potential animal model. Although it is hard to completely meet all of these three criteria, there are a number of good and validated animal models for chronic stress.

Among the most commonly used animals for chronic stress research are rodents and non-human primates. Especially the latter are often used to model diseases, which are likely to be specific to animals with higher brain development (like e.g. depression). The obvious advantage of non-human primates is their close relationship to humans. Their brains share structural and functional features with the human brain and non-human primates display many similar behavioural domains. With these animals it seems most likely that complex diseases like depression can be mimicked. Primate models almost exclusively use social stressors and have generally focused on phenotypes of psychiatric diseases. The social stressor is often based on maternal separation paradigms (Rosenblum and Paully, 1987; Gilmer and McKinney, 2003). Harlow and Suomi have pioneered this work by using maternal separation paradigms in rhesus monkeys to induce depression-like symptoms in

the offspring (Harlow and Suomi, 1971; Harlow and Suomi, 1974). Besides face and construct validity, these models also have shown predictive validity, as the effects could be reversed by antidepressant treatment (Suomi et al., 1978). Another very interesting non-rodent animal model for depression-like symptoms is the tree shrew. First developed by von Holst and colleagues (Von Holst, 1977), male tree shrews are subjected to chronic social defeat stress over a time period of 24 days. Defeated animals show clear symptoms resembling a depression-like state, which can be ameliorated by antidepressant treatment (Meyer et al., 2001). However, the main obstacle with primate studies remains the low availability of test animals due to the high maintenance costs and the obviously involved ethical issues.

To avoid these limitations, rodent models have become a widely accepted alternative to study disease mechanisms for chronic stress. In the past decades, numerous animal models for chronic stress in rodents have been developed. Based on the assumption that chronic stress related diseases are based on elevated levels of glucocorticoids, paradigms of chronic stress often apply physical stressors as repeated restraint or immobilisation stress (Kim and Han, 2006; Sabban et al., 2006). In order to avoid adaptation and predictability of a repeated exposure to the same stimulus, Willner et al. developed the chronic mild stress paradigm (CMS). This nowadays widely used and accepted paradigm for chronic stress in rats or mice utilizes a wide variety of mild physical stressors such as cold, food deprivation, cage tilt or changes in the dark/light cycle with a daily variation of the stressful stimulus (Willner et al., 1987). The phenotype of animals submitted to CMS indeed resembles aspects of depression in many features, including anhedonia (a loss of interest in pleasurable activities), increased immobility in the forced swim test, decreased sexual behaviour, decreased grooming and decreased REM sleep (Willner, 2005). One of the oldest and most widely used animal models is the learned helplessness model (Seligman and Beagley, 1975; Seligman and Beagley, 1975), where the animals are exposed to aversive stimuli, which they can not predict, control or avoid leading to deficits in escape performance, called learned helplessness. Additionally symptoms like loss of appetite and weight as well as a decrease in motor activity occur, resembling those of depressed patients. The kind of stressor used is very critical, though, as different stressors activate different neuronal circuits (Herman and Cullinan, 1997) and marked differences in reactions to physical compared to psychological stressors have been observed.

In humans, the most common stressors reported as risk factors for stress-induced diseases are of social nature (Brown and Prudo, 1981). To increase the construct validity in terms of

the nature of the stressor, other rodent models are based on social stressors. Social stress is probably one of the most pervasive stressors in humans and social animals (Sachser et al., 1998; Bartolomucci et al., 2005; Fuchs, 2005). Animals exposed to inescapable social stress situations for a long time show extreme increases of their hypothalamic-pituitary-adrenal (HPA) system activity and a high mortality rate (Fuchs and Flugge, 2003). The most commonly used experimental paradigms for social stress are the *chronic social defeat* paradigm, where the animals are repeatedly and continuously confronted with a larger and aggressive resident mouse (for review: Koolhaas et al., 1997a), the *visible burrow system*, where in mixed-sex rat groups maintained in a habitat providing burrows and an open area, offensive and defensive behaviors of males are associated with the development of dominance hierarchies (Blanchard et al., 1995; Albeck et al., 1997) or other forms of social instability (Ahima et al., 1998; Ader, 1969; Kudryavtseva, 2000). All of these models show a high degree of face and predictive validity. However, a major drawback of these paradigms is that they are relatively work intensive and space consuming so that only a few animals per group can be tested in a specific experiment. This is of particular importance in view of individual differences in the susceptibility to chronic stress and stress-induced pathologies due to genetic or epigenetic variability. The possibility to examine individual variations within a treatment group, to identify vulnerable and resistant animals and to examine long-lasting changes of the stress experience is thus largely restricted with these paradigms. Another important issue that needs to be addressed when regarding face validity is the persistence of symptoms resulting from chronic stress exposure. The majority of studies investigated only effects during or right after cessation of the stressor, thereby concentrating mainly on acute effects. However, in humans the maladaptive responses to chronic stress often outlast the actual stress phase by many years or even decades. Only few preclinical studies assessed potential long-term effects of acute or chronic stressors and only a small number of studies have pointed out that some of the observed neuroendocrine and behavioral effects evoked by chronic stressors persist after the stress is discontinued, which is a crucial factor with respect to human pathology (Tsankova et al., 2006).

1.3 Hypothalamo pituitary adrenal axis regulation

Every threat to the body, no matter if real or imagined, acts as a stressor and evokes a stress response. This serves to restore homeostasis, a dynamic equilibrium that is strived to be maintained by all living organisms. The process of reestablishing homeostasis in the face of a challenge was termed allostasis (Sterling and Eyer, 1981). It means that stability is

achieved through change, in part by the process of increasing sympathetic and hypothalamo pituitary adrenal (HPA) activity. Thereby mediators such as adrenaline and cortisol are released, which promote adaptation (McEwen and Stellar, 1993; Sterling and Eyer J, 1988). Hence, a brief period of controllable stress is rather beneficial to health. In contrast, lack of control and uncertainty can produce a chronic state of hyper activation of the stress axis where the stress mediators are not turned off adequately. In case of an inadequate or extremely prolonged stress response, where the allostatic systems remain active over a long period of time, the costs of reinstating homeostasis might be too high. This condition is termed allostatic load or overload (McEwen and Stellar, 1993; McEwen, 1997; Schulkin et al., 1994) and is generally considered to enhance vulnerability to disease. Allostatic load leads for instance to impaired immunity, atherosclerosis, obesity, bone demineralization and atrophy of nerve cells in the brain. Many of these processes are seen in major depression and in anxiety disorders (for review see: McEwen, 2004).

Essential to the stress response is, besides the activation of the fast sympathico adrenomedullary system driving the 'fight or flight' response, the activation of the hypothalamo pituitary adrenal axis, which is more persistent in its actions (for review: de Kloet et al., 2005). When the parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus are stimulated, they secrete the peptides corticotropin releasing hormone (CRH) and vasopressin (AVP), which are stored at the nerve terminals in the median eminence, into a portal vessel system that links the median eminence with the anterior part of the pituitary. There they bind to their respective receptors (CRHR1 and V1b) and activate the synthesis of pro-opiomelanocortin (POMC), which is then further processed to corticotropin (ACTH), opioid and melanocortin peptides, among others. CRH is thought to be the main activator of ACTH release, but AVP, which is co-expressed and co-secreted mainly after prolonged stress periods, can amplify the effects. ACTH is released in the blood stream and stimulates via binding to its specific receptors in the adrenal cortex the secretion of cortisol (in humans) and corticosterone (in humans and rodents). Circulating glucocorticoids can reach every organ of the body, thereby contributing to recovery and adaptation. In the periphery they exert their functions mainly via mobilizing energy resources, dampening of immune reactions and increasing the vascular tone (Munck and Naray-Fejes-Toth, 1992). Further, they prevent stress responses from overshooting by affecting specific afferent pathways involved in activating the PVN, such as for example the prefrontal cortex, the hippocampus, nuclei of the brain stem or the amygdala, altogether leading to a reduced HPA axis activity. In addition, glucocorticoids exert a direct negative

feedback at different levels of the HPA axis like the PVN and the pituitary, thereby leading to a normalization of HPA axis function.

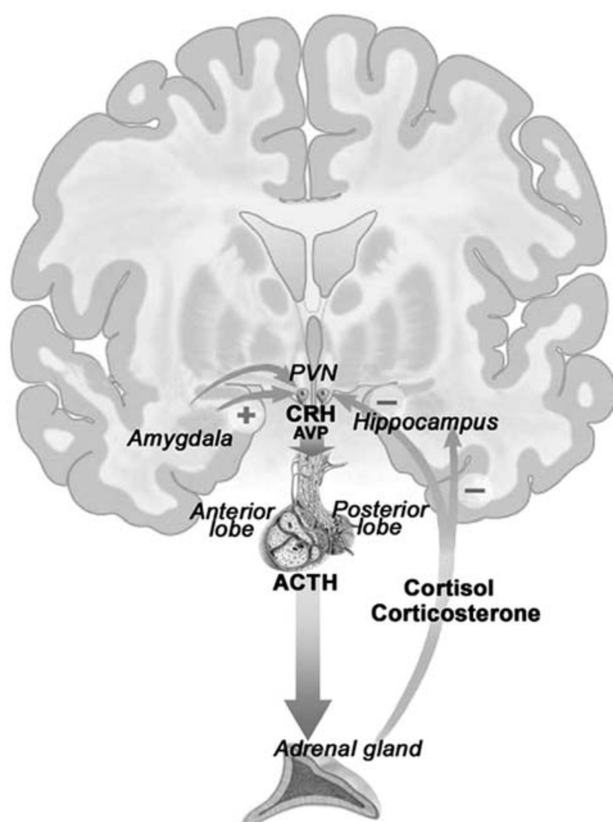

Figure 1: Schematic overview of the hypothalamic-pituitary-adrenal axis (HPA axis) in the neuroendocrine stress response. Activation of the paraventricular nucleus (PVN) of the hypothalamus by the brainstem and higher brain areas initiates the release of corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) at the level of the median eminence into the hypophysial portal vessels. At the anterior lobe of the pituitary CRH and AVP act synergistically to release adrenocorticotropic hormone (ACTH) into the blood stream. ACTH, in turn, stimulates the secretion of glucocorticoids from the fasciculate zone of the adrenal cortex. Corticosteroids feed back to the HPA system by binding to the glucocorticoid (GR) and mineralocorticoid (MR) receptors in the brain and the pituitary to restrain the activity of the HPA axis and restore homeostasis.

Glucocorticoids bind to two related receptors in the brain, the mineralocorticoid receptors (MRs) and the glucocorticoid receptors (GRs), albeit with a different affinity. MRs bind corticosterone with a tenfold higher affinity compared to GRs (Reul and de Kloet, 1985; Arriza et al., 1988) and are largely saturated at low basal corticosterone levels. GRs are additionally activated when corticosterone levels are high, i.e. at the circadian peak or after stressful events. Therefore MRs are implicated in the maintenance of basal activity of the stress system by proactive feedback, while GRs are rather involved in the termination of the stress induced HPA activation by reactive feedback (for review: de Kloet et al., 1998). MRs and GRs have also a distinctly different distribution throughout the brain: MRs are predominantly present in the limbic system with a very dense expression in the hippocampus, whereas GRs are ubiquitously expressed but are most abundant in the hippocampus, hypothalamic CRH neurons and pituitary corticotropes. Co-expression is therefore restricted to a few distinct areas of the limbic system with the hippocampus being the most relevant region. Both receptor types are cytosolic receptors that, upon binding of their ligand, translocate to the nucleus and bind as homo- or -in regions of coexistence of

GR and MR- also as heterodimers to a consensus sequence of the DNA, the glucocorticoid response elements (GRE). Thereby they recruit either co-activators or co-repressors, leading to either activation or repression of gene expression (Meijer et al., 2005). As monomers GRs can also directly interact with transcription factors like NFκB, AP-1 or CREB, thereby leading to a reduction of their transcriptional activity (de Kloet et al., 2005). In addition to the genomic actions of these receptors, MRs have recently been implicated in fast, non-genomic glucocorticoid actions. These pathways are likely to involve MRs inserted into the plasma membrane, which emerge with high corticosteroid concentrations that occur after stress exposure. Therefore the MRs probably shuttle from the cytoplasm to the cell membrane and, following ligand binding, enhance glutamate transmission. Through the fast non genomic and the delayed genomic effects, corticosterone can alter hippocampal activity over a very long period of time during and after experience of a stressful situation (Karst et al., 2005).
Additionally, GRs and MRs have been implicated in the regulation of anxiety related behavior (Gass et al., 2001) and are involved in specific aspects of information processing such as in the modulation of learning and memory (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Tronche et al., 1999; Oitzl et al., 2001). Thereby MRs seem to play a role in the behavioral reactivity during novel situations, while GRs are more important for information storage and consolidation (Oitzl et al., 1994; Oitzl et al., 1997; Akagi et al., 1997; Shors, 2001).
The balance between MRs mediating the maintenance of homeostasis and GRs promoting recovery is therefore very critical for the effects glucocorticoids exert on the brain, leading to a specific tone of HPA axis feedback control (de Kloet, 2003).
In conclusion, the stress response, when activated for a short time, provides the body a fast and efficient way to mobilize energy and to pass through a stressful situation. A prolonged state of hyper activation accompanied by the resulting chronically elevated glucocorticoid levels, though, is maladaptive, rather damaging to the body and is considered to enhance disease vulnerability.

1.4 Scope of the thesis

In avoidance of the before mentioned limitations, a novel mouse paradigm for chronic social stress was developed. In contrast to previous animal models of social stress, which are often based on the differentiation of dominant and subordinate animals (with the latter ones generally found as the more vulnerable animals), this paradigm is based on a complete

disruption of social hierarchy. In order to achieve this situation, the cage composition of male mice housed in groups of four is randomly changed twice per week for a period of seven weeks starting at postnatal day 28. This means, that every mouse in the paradigm is faced with three unknown mice every three to four days, which creates a highly unstable and unpredictable social environment for all mice. Control animals are also housed in groups of four, but remain with the same cage mates throughout the experiment and generally establish a stable hierarchy. At the end of the chronic stress phase all animals are separated and since then kept in single housing. This stress paradigm can be easily conducted with a high number of animals.

In order to appraise the novel model, the following objectives were defined:
(1) validation of the recently developed chronic social stress paradigm with regard to face, predictive and construct validity
(2) investigation of potential persisting or long-term effects of chronic stress during adolescence on a physiological, neuroendocrine and behavioral level
(3) investigation of a potential influence of the stress regimen during adolescence on learning and memory in aged animals
(4) investigation of chronic stress during adolescence as a risk factor for metabolic alterations at older age
(5) assessment of the potential efficacy of treatment with the cannabinoid receptor 1 antagonist rimonabant in preventing consequences of chronic stress

To pursue these objectives, the following experiments were conducted:
In project 1 we characterized the physiological, neuroendocrine and behavioral alterations obtained 7-14 days after cessation of the stress regimen, i.e. after 7-14 days of recovery. Additionally, the efficacy of treating the animals during the stress phase chronically with either the serotonin reuptake inhibitor (SSRI) paroxetine or the corticotropin releasing hormone receptor 1 (CRHR1) antagonist DMP696 on preventing negative consequences evoked by the chronic social stress regimen is assessed. Project 2 characterizes persistent physiological, neuroendocrine and behavioral alterations obtained 12 months after stress termination in comparison to the immediate effects obtained at the end of the stress period. In project 3 we investigated the learning and memory performance of 15 months old animals (i.e. 12 months after cessation of the stressor) on a behavioral, electrophysiological and neurogenetic level. The experiment described in project 4 was performed to assess

metabolic alterations following chronic stress in aged animals. Therefore the body fat distribution of subcutaneous and visceral fat was determined in 12 months old mice using functional magnet resonance imaging In project 5 the impact of chronic rimonabant treatment during the chronic stress period on physiological, neuroendocrine and behavioral alterations observed in untreated stress animals is assessed 7-14 days after cessation of the stress regimen, i.e. after 7-14 days of recovery. To further characterize potential changes in metabolic parameters, at the age of six and twelve months, respectively, the animals were subjected to an oral glucose tolerance test and their glucose and corresponding insulin levels were measured.

Due to the heterogeneity of the data each project will be introduced and discussed separately.

II Material and methods

2.1 Animals and stress procedure
2.2 Drugs
2.3 Sampling procedures
2.4 Analytics
2.5 Behavioral tests
2.6 Electrophysiology
2.7 Magnet resonance imaging
2.8 Statistics

2.1 Animals and stress procedure

Animals

Experiments were carried out with male CD1 mice from the Charles River Laboratories (Maastricht, the Netherlands). The animals were 26-28 days old on the day of arrival. All animals were housed in groups of four per cage (45 x 25 x 20 cm) under a 12L:12D cycle (lights on at 6.00 h) and constant temperature (23 $\pm$ 2 °C) conditions. Food and water were provided ad libitum. The experiments were performed at the animal facility of the Max Planck Institute of Psychiatry in Munich, Germany.
The experiments were carried out in accordance with European Communities Council Directive 86/609/EEC. All efforts were made to minimize animal suffering during the experiments. The protocols were approved by the committee for the Care and Use of Laboratory Animals of the Government of Upper Bavaria, Germany.

Chronic stress procedure

The chronic stress procedure was performed during the adolescent and young adult period of the mice, between the age of 4 and 12 weeks. After a habituation period of 5 days following arrival, the group composition in each cage was changed twice per week for 7 weeks so that four mice from different cages were put together in a new, clean cage. The rotation schedule was randomized to minimize the likelihood of a repeated encounter of the same mice throughout the experiment. Animals of the control group remained with the same cagemates throughout the experiment. After 7 weeks of chronic stress procedure, all mice (control and chronic stress) were separated and single housed for at least seven days before testing (cage size 45 x 25 x 20 cm). Although single housing represents a stressor in itself in many species, in male mice it has been shown that single housing does not affect main immuno-endocrine parameters under basal conditions (Bartolomucci et al., 2003b). Body weight was monitored every two weeks throughout the stress procedure. Animals with bites were excluded from the experiments (less than 2% of the animals in the chronic stress group).
For the investigation of long-term consequences, all mice (control and chronic stress) were separated at the end of the stress procedure and since then kept in single housing (cage size 45 x 25 x 20 cm) until conduction of the respective experiments.

Determination of social status

The social status and interactions of the animals in the chronic stress and in the control group were determined according to literature reports (Bartolomucci et al., 2004), with some adaptations. Each cage was observed soon after regrouping (stress animals) or cage change (control animals) for an observation period of 60 minutes. During this time measurements of aggression, grooming, resting and food intake were taken every 5 minutes for 1 minute (12 observation periods in total). The animal chasing and biting the other cage mates was identified as dominant.

2.2 Drugs

All drugs were applied via drinking water. Paroxetine was obtained from GlaxoSmithKline (Munich, Germany) as a solution and was diluted in tap water to a final concentration of 0,16 mg/ml. With average water consumption of 5 ml/mouse/day, the daily dose of paroxetine was approximately 20 µg/g body weight. Fluid intake was monitored daily and the variation of fluid intake was found to be less than 10% over the course of the experiment, thereby ensuring the correct dosage of the drug. The CRHR1 antagonist DMP696 was donated by Bristol-Myers Squibb (New York, USA). The drug was dissolved in a 0,25% Methocel (Georg Breuer GmbH, Germany) solution at a stock concentration of 16 mg/ml by bead milling it over night at 4 °C. The stock solution was then diluted with water to a final concentration of 0,16 mg/ml (approximately 20 µg/g body weight per day). The CB1 antagonist rimonabant (SR141716A) was donated by Sanofi-Aventis (Bagneux, France). The drug was dissolved in 100% EtOH by vortexing for 10 minutes and after adding of 1 ml water by bead milling 2 hours at room temperature (stock concentration of 24 mg/ml). The stock solution was then further diluted with water to a final concentration of 0,08 mg/ml and applied via the drinking water. With average water consumption of 5 ml/mouse/day, the daily dose of rimonabant was approximately 10 µg/g body weight. Fluid intake was monitored daily and the variation of fluid intake was found to be less than 10% over the course of the experiment, thereby ensuring the correct dosage of the drug.
The respective dosages of paroxetine, DMP696 and rimonabant were based on literature reports (Keck et al., 2003; Li et al., 2003; Griebel et al., 2005). Drug solutions were replaced on a daily basis.

2.3 Sampling procedures

Trunk or tail cut blood was collected individually in labeled 1.5 ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Germany). The time between the first disturbance of the animals and the sampling was in all cases less than 1 minute. Tail blood was collected without anesthesia as described previously (Fluttert et al., 2000) by inflicting a small incision at the bottom of the mices' tail using a racor blade. For trunk blood collection animals were anesthetized in isoflurane and decapitated. All blood samples were kept on ice and later centrifuged for 15 minutes at 6000 rpm at 4 °C. Plasma was transferred to clean, labeled 1.5 ml microcentrifuge tubes. All plasma samples were stored frozen at –20 °C. After decapitation, brains were removed, frozen in isopentane at –40 °C and stored a –80 °C for in-situ hybridization. Adrenal and thymus glands were removed, dissected from fat and weighed.

2.4 Analytics

In situ hybridization

Frozen brains were sectioned at -20 °C in a cryostat microtome at 16 µm in the coronal plane through the level of the hypothalamic PVN and dorsal hippocampus. The sections were thaw-mounted on superfrost slides, dried and kept at -80 °C. In situ hybridization using 35S UTP labeled ribonucleotide probes (CRH, GR, MR, GluR1, α1GABA(A) and NMDA NR1) were performed as described previously (Schmidt et al., 2002). Briefly, for riboprobe in-situ hybridization sections were fixed in 4% paraformaldehyde and acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine/HCl. Subsequently, brain sections were dehydrated in increasing concentrations of ethanol. The antisense cRNA probes for CRH, GR, MR, GluR1, α1GABA(A) and NMDA NR1 were transcribed from the respective linearized plasmid. Tissue sections were saturated with 100 µl of hybridization buffer containing approximately 1.5 x 10^6 cpm 35S labeled riboprobe. Brain sections were coverslipped and incubated overnight at 55 °C. The following day the sections were rinsed in 2 X SSC (standard saline citrate), treated with RNAse A (20 mg/l) and washed in increasingly stringent SSC solutions at room temperature. Finally sections were washed in 0.1 X SSC for 1 hour at 65 °C and dehydrated through increasing concentrations of alcohol. For AVP and BDNF, oligo in-situ hybridizations were performed. The oligonucleotides (sequence: 5' gggcttggcagaatccacggactcttgtgtcccagcc-gctgtaccag 3' for AVP and 5'agttccagtgccttttgtctatgcccctgcagccttccttggtgt3' for BDNF, respectively) were labeled with

35S dATP using terminal transferase (TdT, Boehringer, Ingelheim, Germany) and added to the hybridization mix. Brain sections were coverslipped and incubated overnight at 45 °C. The following day the sections were washed in 1 X SSC at 55 °C and dehydrated through increasing concentrations of alcohol.

The slides were apposed to Kodak Biomax MR films (Eastman Kodak Co., Rochester, NY) and developed. Autoradiographs were digitized, and relative expression was determined by computer-assisted optical densitometry (Scion Image, Scion Corporation, Frederic, USA). The mean of 4 measurements of two different brain slices was calculated from each animal. The data were analyzed blindly, always subtracting the background signal of a nearby structure not expressing the gene of interest from the measurements. For AVP, slides were also dipped in Kodak NTB2 emulsion (Eastman Kodak Co., Rochester, USA) and exposed at 4 °C for 6 days. Slides were developed, counterstained with cresyl violet staining and examined with a light microscope with both bright and dark field condensers.

Radio immuno assay

For quantitative analysis of corticosterone and ACTH in plasma a commercial radioimmunoassay (RIA) kit (MP Biomedicals Inc. (Illkirch, France); sensitivity 6.25 ng/ml and 10 pg/ml, respectively) was used according to the instructions of the manufacturer's protocol. Corticosterone and ACTH were measured in the same animals.

Western blot

The hippocampus was homogenized in HEPES buffer containing 1% NP40 and several proteinase inhibitors (based on Hope et al., 1994), and centrifuged to eliminate cell debris. The supernatant was used as total protein sample. Protein concentration was determined with the BioRad DC protein kit (BioRad, München, Germany). 25 µg protein per lane were loaded on 9% SDS–PAGE and transferred to nitrocellulose (Protran BA85, 45 µm, Schleicher and Schüll, Dassel, Germany), using a Mini Transfer Cell (BioRad). The membranes were blocked with 5% BSA in TBS containing 0.1% Tween 20 (TBS-T) and incubated with the different primary antibodies overnight. The following antibodies were used for Western blot analysis: NMDAR2B (Chemicon, Upstate), GluR1 (AB06-306 Upstate) and α1-GABAA (AB06-868, Upstate). Incubation with the secondary antibody (horseradish peroxidase-conjugated donkey anti-rabbit antibody, Amersham Buchler, Braunschweig, Germany) lasted 2 hours. All antibody incubations, washes and dilutions were performed in TBS-T. Antibody detection was performed with Amersham ECL

Western blotting analysis system according to the manufacturer's protocol. ECL signal was exposed to Hyperfilm-ECL (Amersham Buchler). For control of protein amounts, samples (25 µg) were run on a 9% SDS–PAGE and the gels were stained with Coomassie Brilliant Blue. One gel was stained with Coomassie Blue and used to normalize the data and ensure equal loading on each well of the gel. To check for efficacy of protein transfer from the gel and to match Coomassie-stained bands with the respective protein levels, reversible staining of the membrane was also done with Ponceau S after transfer to ensure homogeneity of transfer. The data were normalized to the amount of protein loaded in each lane of the Coomassie Blue stained gel that was run in parallel by measuring the intensity of the whole lane.

Unless otherwise stated all chemicals were obtained from Sigma (Deisenhofen, Germany). At least 3 blots were prepared per antibody which were analyzed and averaged. Each Western blot comprised the control and the remaining experimental group. Blot autoradiographs were quantified by computer-assisted densitometry using the Optimas image analysis system (Optimas/BioScan, Edmonds, USA). All data are expressed as relative grey values and the values for the different groups were determined by setting the control group to 100% and calculating the relative percentages of the other groups. The data for the different groups were normalized to the amount of protein loaded in each lane in the Coomassie blue-stained gel that was run in parallel.

2.5 Behavioral tests

All behavioral tests were performed between 8.00 and 12.00h in the room where the animals were housed.

Open field test

Open field arenas (50 x 50 x 50 cm) were made of gray PVC and evenly illuminated during testing (20 lux). General locomotor activity was recorded for 30 minutes (distance traveled) using a video-tracking system (Anymaze 4.20, Stoelting, Illinois, USA). The locomotor activity of the first and the fifth minute of open field exposure were subtracted to calculate the locomotor adaptation in the novel environment as a measure of anxiety. A high degree of locomotor adaptation indicates low anxiety, while a low degree of locomotor adaptation indicates high anxiety. The last 20 minutes of the open field test were used to assess differences in general locomotor activity between the groups.

Novelty-induced suppression of feeding paradigm

The test was performed according to Merali et al. with some adaptations (Merali et al., 2003). 3 days before testing the animals received an almond sliver in a small plastic dish in their home cage for 2 consecutive days. The consumption time of the almond in the home cage environment was less than 30 seconds for all mice on the second day, with no differences between the groups. Testing was performed in an open field arena as described above, with a plastic dish in the center containing a small piece of almond. The animals were familiar with both the dish and the almond, but were unfamiliar with the open field arena. The latency until the initiation of food intake was recorded. The total test time was 30 minutes. Shorter consumption times were interpreted as lower anxiety levels.

Elevated Plus maze

The elevated plus maze consisted of two opposing open arms (30 x 5 x 0,5 cm) and two opposing enclosed arms (30 x 5 x 15 cm) of gray PVC, which were connected by a central platform (5 x 5 cm) shaping a plus sign. Animals were placed in the center of the plus maze and were allowed to explore it for 5 minutes. Percent of time spent in the open arms and percent entries to the open arms were recorded, as well as head dips (Rodgers and Dalvi, 1997; Müller et al., 2003).

Y-maze

The Y-maze was made of grey PVC and consisted of three arms with an angle of 120° between each of the two arms differentially marked by tape symbols (triangles, bars and plus-signs, respectively). The arms were 30 x 10 x 15 cm (l x w x h) each and evenly illuminated during testing (40 lux). The Y-maze test comprises two trials separated by an intertrial interval (ITI) to assess spatial recognition memory. During the first trial, the acquisition phase, the mouse was allowed to explore two of the three arms for 5 minutes while the third arm was blocked. After an ITI of 1 hour the second trial, the retrieval phase, was conducted during which all three arms were accessible for 5 minutes. The percentage time spent in the novel arm compared to the known arms was scored using a video-tracking system (Anymaze 4.20, Stoelting, USA) with a significantly higher percentage than chance level (>33,3 %) being rated as successful spatial memory.

Social discrimination task

The test was performed according to Engelmann et al. with some adaptations (Engelmann et al., 1995). All test animals were habituated to the experimental cages for 40 minutes followed by a 5-minute presentation of an ovarectomized female, which was placed into the cage immovable in a perforated plastic tube. After an intertrial interval of 1 hour, the first, familiar female was reintroduced to the male test mouse together with a novel, unfamiliar female. The time spent for olfactory investigation of the novel stimulus-female compared to the familiar one was observed. Olfactory investigation time of the unfamiliar female was taken as a parameter for the animals' social memory.

Object recognition test

Testing was performed in an open field box (50 x 50 x 50 cm) under moderate illumination (30 lux). The test consisted of two phases, the sample phase and the trial phase. After habituation of 15 minutes, where the animals were allowed to become familiar with the empty arena, the test subjects were presented a glass object (e.g. a salt shaker) which they could freely explore for 5 minutes (sample phase). After an intertrial interval of 1 hour, where the mice were returned to their home cages, they were again placed into the arena and presented two different objects, the familiar glass one and a novel metal one, similar in size. To avoid influences of spatial memory, the location of the objects was changed between sample and trial phase. The percentage of the number and the duration exploring the unknown object compared to the known object was calculated, with a higher preference for the novel object being rated as intact recognition memory.

Morris water maze

The test was performed according to Morris with some adaptations for mice (Morris, 1984). Briefly, a circular pool (80 cm in diameter) was filled with 26 $\pm$ 1 °C water that was made opaque by addition of chalk. A white painted platform (9 cm in diameter) was placed inside the pool 1 cm above the surface (visual training) or 1 cm below the surface of the water (spatial training). Several extra-maze visual cues were attached to the walls of the experimental room, approximately in a distance of 50-100 cm from the pool. After 1 day of a free swim trail, the 3 following days the mice were put on the platform for 1 minute each (pre-training). On day 5 they had to swim, locate the visible platform and climb onto it (visual training). In the spatial training phase the animals had to perform 4 trials each day on 3 consecutive days (day 8-10), where the now non-visible platform remained in a

constant position over the trials and days. The latency to reaching the platform as well as the total distance traveled was analyzed using a video-tracking system (Anymaze 4.30, Stoelting, USA).

2.6 Electrophysiology

Transverse hippocampal slices (350 µm) were obtained from the brains of adult, 15-month-old mice, that were anesthetized with isoflurane and then decapitated. The brain was rapidly removed, and slices were prepared in icy Ringer solution using a vibroslicer. All slices were placed in a holding chamber for at least 60 minutes and were then transferred to a superfusing chamber for extra cellular or whole-cell recordings. The flow rate of the solution through the chamber was 1,5 ml/min. The composition of the solution was 124 mM NaCl, 3 mM KCl, 26 mM NaHCO3, 2 mM CaCl2, 1 mM MgSO4, 10 mM D-glucose, and 1,25 mM NaH2PO4, bubbled with a 95% O2–5% CO2 mixture, and had a final pH of 7,3. All experiments were performed at room temperature.

Extra cellular recordings of field excitatory postsynaptic potentials (fEPSPs) were obtained from the dendritic region of the CA1 region of the hippocampus using glass micropipettes (1–2 MΩ) filled with superfusion solution. For LTP induction, high-frequency stimulation conditioning pulses (100 Hz/1 s) were applied to the Schaffer collateral-commissural pathway. Measurements of the slope of the fEPSP were taken between 20 and 80% of the peak amplitude. Slopes of fEPSPs were normalized with respect to the 20-min control period before tetanic stimulation.

fEPSPs were evoked by stimuli (0.033Hz, 4–5 V, 20 µs), delivered via two bipolar tungsten electrodes insulated to the tip (5-µm tip diameter) and positioned in the Schaffer collateral–commissural pathway. The recordings were amplified, filtered (3 kHz) and digitized (9 kHz). The digitized responses were stored to disk using a data acquisition program.

Stimulation, recording and analysis of excitatory postsynaptic potentials and LTP in the CA1 region of the hippocampus were performed as described previously (Simon et al., 2001).

2.7 Magnet resonance imaging

To assess intra abdominal fat distribution we applied magnetic resonance imaging. The animals were measured ex-vivo on a 7 T BRUKER Biospec system, equipped with a volume resonator to allow for whole-body imaging of the mouse. After the animals had been decapitated the cadavers were frozen at -20 °C for later use. For MR-imaging,

cadavers were thawed for 8 hours, measured in a prone position and fixated with tape. A T1-weighted sequence was performed (2D spin echo, TR = 900 ms, TE = 11.6 ms, matrix size 512 x 256, slice thickness = 1 mm, 34 slices, in plane field of view 4.6 cm x 4.6 cm, 8 averages, total acquisition 30 min). Using this sequence, MR-images provide a strong contrast between fat and muscular/organ tissue. In addition, as compared to T2-weighted images, free liquids also display low intensity and can therefore be clearly differentiated from fat. To achieve full body coverage, two slice packages with one slice overlap were scanned subsequently without repositioning of the mouse, covering the complete abdomen from tail to liver.
Anatomical images were analyzed using the ParaVision software (BRUKER,Germany). For each slice, regions of interests were set including visceral tissue, and excluding subcutaneous, cutaneous and skeletal muscle compartments. The fat compartment was analyzed based on these regions along with an image intensity threshold. To account for the potential variability of absolute image intensities in different slices, the image intensity of fat was determined per each slice using an intensity histogram, identifying the maximum of the fat peak distribution. Based on this value, an absolute lower threshold was calculated corresponding to 2/3 of fat peak intensity. With this threshold, fat intensities could clearly be distinguished from other tissue components. Cutaneous and subcutaneous fat compartments were identified by summing all image pixels above the threshold excluding visceral volumes. Total fat was estimated by summing the areas of the respective fat compartment in all slices. Subsequently, relative ratios of visceral and subcutaneous fat were calculated.

2.8 Statistics

The commercially available program SPSS 12 was used for statistical analysis. Comparisons of two conditions were made by unpaired t-test or in case of fur condition by the non-parametric Mann-Whitney-U test. When more than two groups were compared a one-way analysis of variance (ANOVA) was used, followed by post-hoc Tukey test. In case of fur condition, a non-parametric Kruskal-Wallis Test was performed, followed by Mann-Whitney-U tests. Morning/evening hormone levels were analyzed with a 2-way ANOVA, with condition as a between factor and time as a within factor. For the analysis of the corticosterone/ACTH ratio the absolute levels were first calculated in percent, with the mean value of the control group set at 100%. For insulin in project 5, in case of one missing observation, missing data were estimated by linear imputation using the remaining

observations as predictors in a multiple regression analysis. At the age of six month these were 9,2% of the data, at the age of twelve months 5,6%. The level of significance was set at $p<0.05$; a trend was accepted with $p<0.1$. Data are presented as mean ± SEM.

III Results

1. Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence
2. Long-term behavioral and neuroendocrine alterations following chronic social stress during adolescence: implications for stress-related disorders
3. Lasting consequences of chronic stress during adolescence on cognition
4. Long-term consequences of chronic stress during adolescence on body fat distribution
5. Rimonabant treatment during chronic stress: behavioral, neuroendocrine and metabolic consequences

1. Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence

Chronic stress is generally considered a key risk factor for the development of a variety of human diseases (Anisman and Zacharko, 1992). Specifically anxiety and depressive disorders have frequently been associated with preceding periods of chronic stress or stressful life events (Post, 1992; Amat et al., 2005). In addition to psychiatric disorders, chronic or severe stress has also been implicated in a variety of other diseases, such as the metabolic syndrome (including diabetes type II), cardiovascular disease and hypertension (Henry and Stephens, 1977; Pickering, 2001; Tennant, 2001).

To study the multiplicity of neurochemical pathways that are affected by chronic stress, a wide range of stimuli and paradigms have been used to induce a state of stress pathology. The more frequently used rodent paradigms for chronic stress often consist of the repeated exposure of the animal to physical stressors, such as electric shock, water immersion or restraint. However, large differences exist in the duration of the stressor, ranging from 2 to 16 hours per day for 7 to 28 days (Ader and Grota, 1970; Ottenweller et al., 1989; Aguilera, 1994; Conrad et al., 1996; Anisman et al., 1998). Despite the intensity of the stressors, many of these paradigms do not fulfill the criteria of chronic stress, as the stressors are only applied for a relatively short time per day or for only a few consecutive days. In order to circumvent the additional problem of habituation and predictability, Willner and colleagues developed a paradigm of chronic mild stress (CMS), where different types of mild stressors are alternated (Willner, 2005). Mice or rats subjected to CMS have been shown to display a variety of behavioral changes mimicking clinical symptoms of depressed patients (Willner, 2005). However, these procedures do not take into account the etiology of human stress-associated disorders, as the majority of stressful stimuli in humans that have been shown to increase the risk of psychiatric disorders are of a social nature (Brown and Prudo, 1981). In an attempt to investigate the neurobiological mechanisms underlying disease susceptibility caused by social stress, it is crucial that the animal model meets the requirements of construct validity and, therefore, investigates the consequences of social stress in rodents (Geyer and Markou, 1995).

Social stress is probably one of the most pervasive stressors in humans and social animals (Bartolomucci et al., 2005; Fuchs, 2005). Animals exposed to inescapable social stress situations for a long time show extreme increases of their hypothalamic-pituitary-adrenal (HPA) system activity and a high mortality rate (Albeck et al., 1997). There are a number

of experimental designs relying on social stress, such as the social defeat paradigm in rats and mice, the sensory contact model in mice or the visible burrow system in rats (Ader, 1969; Blanchard et al., 1995; Albeck et al., 1997; Koolhaas et al., 1997b; Ahima et al., 1998; Stefanski, 2000; Aguilera et al., 2001; Stefanski, 2001; Veenema et al., 2003). A number of other species have also been extensively studied with regard to the behavioral and neuroendocrine consequences of chronic stress and social status, such as the guinea pig, the marmoset or the squirrel monkey (Sachser et al., 1998; Dettling et al., 2002; Levine and Mody, 2003; Parker et al., 2005). Few studies have also indicated that some of the observed neuroendocrine and behavioral effects evoked by these stressors persist even if the stress is discontinued, which is a crucial factor with regard to human pathology (Tsankova et al., 2006). A major drawback of these paradigms is that they are relatively work intensive and space consuming so that only a few animals per group can be tested in a specific experiment. This is of particular importance in view of individual differences in the susceptibility to chronic stress and stress-induced pathologies due to genetic or epigenetic variability.

An important characteristic of chronic stress paradigms is the age, at which the animals are exposed to the stressor. There are many indications that across the life span there are specific windows of vulnerability, where high levels of stress have an increased impact on further development (Heim and Nemeroff, 2001; Spencer et al., 2006; Avital et al., 2006). Many of the available chronic stress paradigms have been tested in young adult mice and rats (about 3 months of age). However, a high vulnerability has also been postulated in the adolescent period, due to the many still ongoing hormonal and neurodevelopmental processes (Tsoory et al., 2007). Especially social stressors are likely to have a high impact during this time as puberty seems crucial for the acquisition of social skills, which form the basis of social interactions and stability during adulthood (Sachser et al., 1998). Thus, the adolescent period is a highly adaptive period, where behavioral and neuroendocrine set points are fine tuned.

In the current studies we therefore developed a novel mouse paradigm, which utilizes chronic social stress as a key pathogenic factor during adolescence. Based on early work by the groups of LeMoal and Henry in rats (Henry and Stephens, 1977; Klein et al., 1992; Arai and Widmaier, 1993), this paradigm creates an unstable social environment for a prolonged period of time. In this paradigm the animals are exposed to a continuous stressful situation, which they cannot escape from and which they are unable to adapt to. Another important advantage is the applicability of this paradigm to a large number of animals. Here we

characterize the paradigm with respect to effects on neuroendocrine and behavioral functions. We report persistent effects of this novel paradigm on stress-related physiological, neuroendocrine and behavioral parameters and their reversibility by a clinical efficacious antidepressant (the selective serotonin-reuptake inhibitor paroxetine) or CRH receptor type 1 (CRHR1) -antagonist treatment.

Experimental design

Experiment 1

In order to assess corticosterone blood levels during the stress procedure, basal morning tail blood samples of 12 control and 12 chronic stress animals were taken on the day before single housing (three days after the last rotation).
For the long-term effects of chronic stress a total of 96 animals (48 control and 48 chronic stress animals) were used. A subgroup of 12 control and 12 chronic stress animals were used for neuroendocrine testing 7 days after the termination of group housing. Evening blood samples were taken between 16.00h and 18.00h by tail cut (Fluttert et al., 2000). The fur state of the animals was rated by two independent researchers according to a 4-point scale (Mineur et al., 2003; Ducottet and Belzung, 2005), with 1 as the best and 4 as the worst fur condition. The next morning, the animals were decapitated under basal conditions. The remaining animals were tested in the following behavioral tests: open field test (after 8-9 days of single housing), plus maze (after 10 days of single housing) and novelty-induced suppression of feeding (after 11-12 days of single housing).

Experiment 2

A total of 152 animals (108 chronic stress, 44 control) were used. 3 weeks after the start of the chronic stress procedure, 36 animals of the chronic stress group were treated with the antidepressant paroxetine and another 24 animals with the CRHR1 antagonist DMP696 for four weeks until the end of the stress procedure. At the start of the drug treatment animals of the chronic stress group showed clear symptoms of stress. After 1 week of single housing with no further drug treatment, 19 control animals, 12 animals of the untreated chronic stress group, 10 animals of the paroxetine treated chronic stress group and 10 animals of the DMP696 treated chronic stress group were used for neuroendocrine testing as described in experiment 1. The remaining animals underwent the same behavioral tests as described in experiment 1. The experimental design is summarized in figure 2.

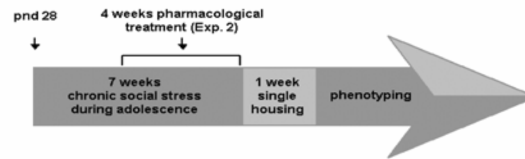

Figure 2: Experimental design.

Results

Experiment 1

Social status and interactions

Social and hierarchical interactions of the animals were determined in a separate set of animals over the first 4 weeks of stress exposure and in control animals. The results of this analysis are presented in table 1. Our data demonstrate that in the chronic social stress group the number of aggressive attacks is significantly higher compared to control animals, already after 1 week of exposure to the stress paradigm. In addition, the latency of an aggressive attack is much shorter compared to control animals. The magnitude of these effects increases over time. Thus, in control animals, a stable social status of the animals developed within the first 2 weeks of group housing. A constant hierarchy without the presence of a female has been shown to represent a non-stressed housing condition and therefore a valid control group (Bartolomucci et al., 2001; Bartolomucci et al., 2004). In contrast, animals exposed to the chronic stress paradigm were not able to establish a stable social environment.

Table 1: Hierarchical interactions

	number of aggressive attacks		latency to first aggressive attack	
	control N = 16	chronic stress N = 16	control N = 16	chronic stress N = 16
week 1	1.25 +/- 0.63	3.25 +/- 3.25	31.25 +/- 9.87	46.25 +/- 13.75
week 2	0.5 +/- 0.5	2.75 +/- 1.11	56.25 +/- 3.75	37.5 +/- 8.29
week 3	0.5 +/- 0.5	6.5 +/- 4.27	55.00 +/- 5.00	35.5 +/- 14.61
week 4	1.0 +/- 0.71	7.5 +/- 3.01	42.00 +/- 10.7	5.5 +/- 1.44

Physiological data

Body weight was determined every other week prior to the cage change. There were no differences between the groups in mean body weight during the experiment ($p<0.65$, t-test). Adrenal and thymus glands of 12 control and 12 chronic stress animals sacrificed after 7

days of single housing were dissected and weighed (table 2). The relative weight of the adrenal glands in chronically stressed animals was significantly increased, while relative thymus weight was significantly decreased (t-test, for p value see table 2).

The fur condition of the 12 control and 12 chronic stress animals was rated carefully by two independent observers according to a 4 point scale prior to blood sampling, with a higher number corresponding to a worse fur state. A significant deterioration of the fur state of chronically stressed animals could be detected (Mann-Whitney U test, Z= -3.089, p=0.002)

Table 2: Long-term effects of chronic stress exposure on adrenal weight, thymus weight and fur state. *significant from control group, p<0.05

	control N = 12	chronic stress N = 12	p value
adrenal weight (mg/g bw)	0.077 ± 0.006	0.123 ± 0.015*	0.008
thymus weight (mg/g bw)	1.388 ± 0.1	0.99 ± 0.109*	0.009
fur state	1.166 ± 0.11	2.091 ± 0.25*	0.002

Neuroendocrine data

Corticosterone basal levels were measured directly at the end of the chronic stress exposure in a separate cohort of animals to validate the effectiveness of the paradigm (figure 3A). Three days following the last cage member rotation, basal levels of animals from the chronic stress group were significantly higher compared to group housed control animals (p<0.05, t-test).

After 7 days of single housing basal evening and morning corticosterone and ACTH plasma levels were measured (figure 3B and 3C). ANOVA with repeated measures (time as within factor, treatment as between factor) revealed a significant effect of time for corticosterone (F (1,21) = 48.148 (p<0.001)) and ACTH (F (1,21) = 14.843 (p<0.01)). No significant interaction between time and condition was observed (corticosterone: (F (1,21) = 3.712 (p<0.068)); ACTH: (F (1,21) = 4.178 (p<0.06)). Morning corticosterone levels are higher in chronic stress animals compared to controls, while ACTH levels are significantly decreased. The analysis of the circadian corticosterone rhythm (difference of morning-evening values) showed that it was flattened in the chronic stress group (p = 0,064). Further, in the morning chronic stress animals had a significantly higher corticosterone/ACTH ratio compared to control animals (figure 3D, p<0.05, t-test).

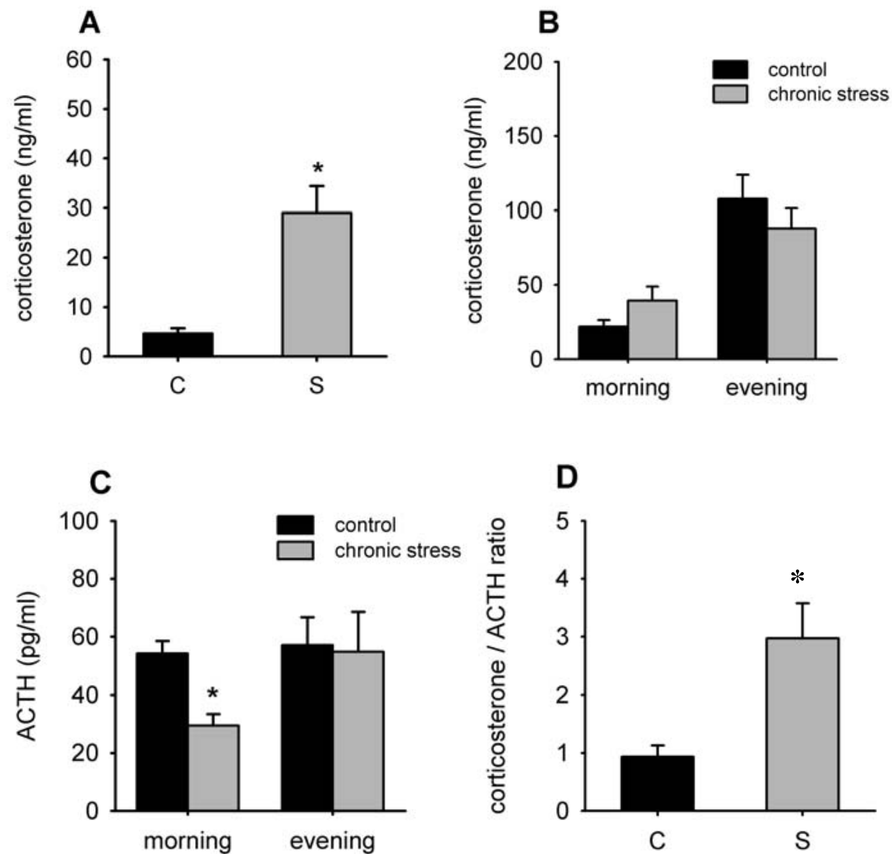

Figure 3: Effects of chronic stress exposure on HPA axis function. (A) Morning corticosterone levels after 7 weeks of chronic stress exposure. (B) Morning and evening corticosterone levels after 1 week of recovery from chronic stress. (C) Morning and evening ACTH levels after 1 week of recovery from chronic stress. (D) corticosterone / ACTH ratio after 1 week of recovery from chronic stress. Control = C; chronic stress = S. N=12 per group. * significant from control group, $p<0.05$

We also analyzed basal gene expression of CRH and AVP mRNA in the PVN, CRH mRNA in the amygdala as well as MR and GR mRNA expression in the hippocampus (figure 4). We found a significant decrease of MR mRNA levels in the CA2 area of the hippocampus in chronically stressed animals ($p<0.05$, t-test). GR mRNA was significantly decreased in the CA1 region ($p<0.05$, t-test). No changes of CRH and AVP expression were observed between the two groups after 7 days of single housing (t-test, $p=0.55$ and 0.23, respectively).

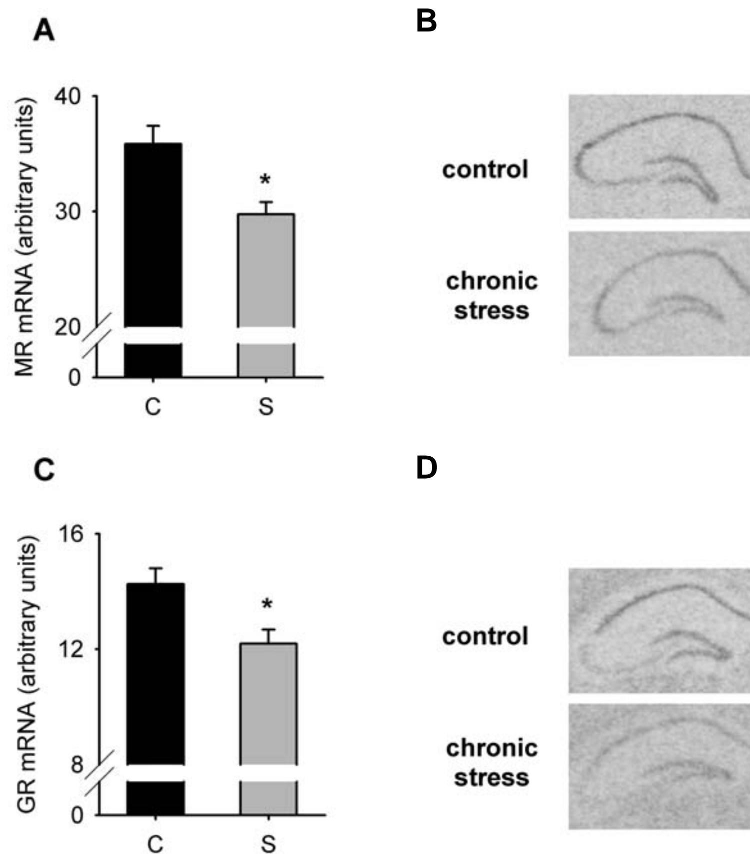

Figure 4: Expression levels of MR mRNA in the CA2 region of the hippocampus (A) and GR mRNA in the CA1 region of the hippocampus (C) in previously chronically stressed and control animals after 1 week of recovery. (B) Representative MR expression autoradiograph. (D) Representative GR expression autoradiograph. Control = C; chronic stress = S. N=12 per group. * significant from control group, $p<0.05$

Behavioral data

To assess long-term alterations in behavior due to chronic stress exposure we tested the animals in anxiety-related behavioral tests. In the open field (figure 5A), the locomotor adaptation in the first 5 minutes was significantly decreased in chronic stress animals ($p<0.05$, t-test). General locomotor activity in the last 20 minutes of the open field exposure was not different in both groups.

Chronically stressed animals also displayed an enhanced anxiety-related behavior in the novelty-induced suppression of feeding paradigm (figure 5B). Control animals initiated the snack consumption significantly faster compared to previously stressed animals ($p<0.05$, t-test).

In the elevated plus maze (figure 5C-D), control animals entered the open arms more often ($p<0.05$, t-test) and spent more time in the open arms compared to animals from the chronic stress group ($p<0.1$, t-test). Further, the number of head dips was significantly reduced in

the chronic stress group (p<0.05, t-test) The number of total arm entries was not different between the groups (p=0.51, t-test).

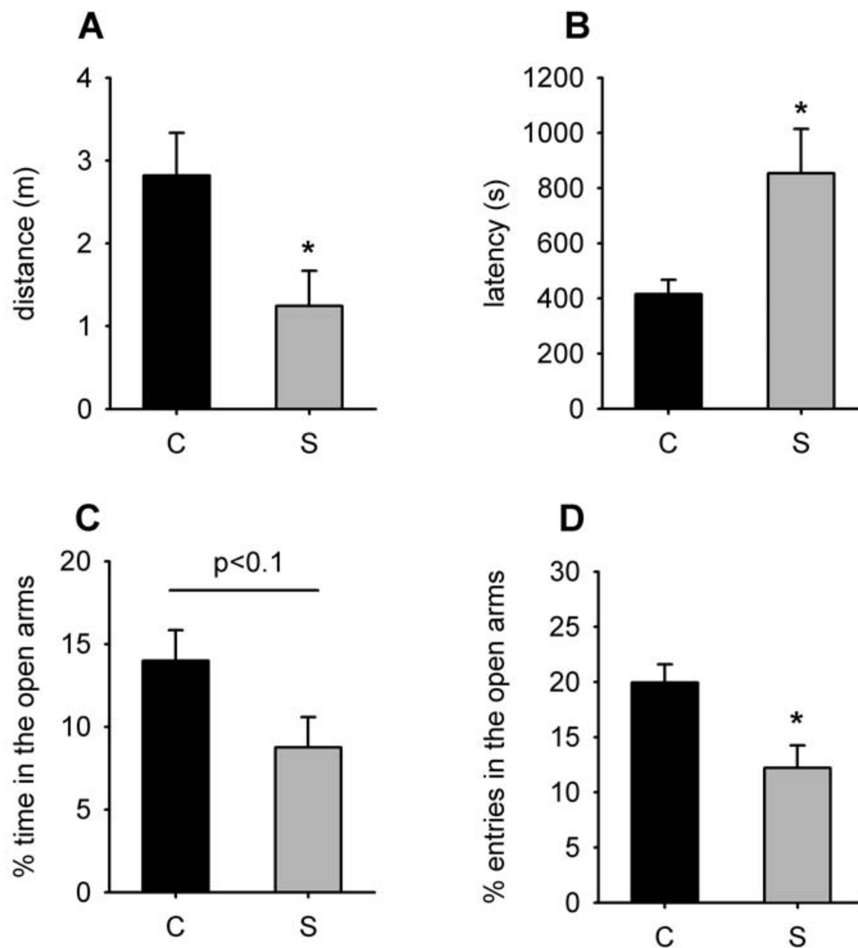

Figure 5: Open field: Long-term effects of chronic social stress on locomotor adaptation during the first 5 minutes of open field exposure (A). Novelty-induced Suppression of Feeding: Effects of chronic stress on the time till initiation of consumption of a palatable snack (B). Plus Maze: Persisting effects of chronic social stress on the time the animals spend in the open arms (C) and the number of entries in the open arms (D). Control = C; chronic stress = S. N=12 per group. * significant from control group, p<0.05

Experiment 2

Physiological data

Body weight was determined every other week prior to the cage change. ANOVA revealed a significant interaction between time and treatment (F (3,96) = 11.602 (p<0.001)). There was no difference in body weight between untreated chronic stress and control animals. Paroxetine, but not DMP696, treatment resulted in a slight but significant increase in body weight after 4 weeks of treatment. Adrenals and thymus glands were dissected and weighed after 7 days of single housing under basal conditions. For both organs, ANOVA revealed an effect of condition (adrenals: F (3,48) = 9,166 (p<0.001); thymus: F (3,50) = 7,100 (p<0.001)) (table 3). Chronic stress resulted in a significant increase in adrenal weight. This effect was prevented by DMP696 or paroxetine treatment during stress exposure. Thymus weight was significantly reduced in the untreated chronic stress group compared to control animals. Paroxetine, but not DMP696, ameliorated this effect.

The fur condition of all animals was inspected carefully by two independent observers according to a 4 point scale (table 3). ANOVA revealed a significant effect of condition (F (3,99) = 21,95 (p<0.001)). Chronically stressed animals scored significantly worse in their fur state compared to control animals. Treatment with either paroxetine or DMP696 did not affect the fur state of the animals.

Table 3: Influence of DMP696 or paroxetine treatments during chronic social stress exposure on adrenal weight, thymus weight and fur state. * significant from control group, p<0.05

	control N = 12	chronic stress N = 12	chronic stress + paroxetine N = 10	chronic stress + DMP696 N = 10	F-value	p value
adrenal weight (mg/g bw)	0.058 ± 0.007	0.096 ± 0.008*	0.049 ± 0.005	0.063 ± 0.007	9.166	0.001
thymus weight (mg/g bw)	1.52 ± 0.057	1.009 ± 0.067*	1.28 ± 0.081	1.183 ± 0.137*	7.1	0.001
fur state	1.31 ± 0.07	2.23 ± 0.13*	2.02 ± 0.07*	2.36 ± 0.12*	----	0.001

Neuroendocrine data

For corticosterone (figure 6A), ANOVA revealed a main effect of condition (F(3,47) = 2.924 (p<0.05)). Morning basal levels of circulating corticosterone were significantly increased in chronically stressed mice. This effect was prevented by treatment with DMP696 or paroxetine during stress exposure. No significant differences were found for ACTH (figure 6B). However, ANOVA revealed a significant effect for the corticosterone/ACTH ratio (F(3,41) = 2.932 (p<0.046)). Chronic stress animals had a

significantly higher corticosterone/ACTH ratio compared to control animals. This effect was abolished by treatment with either paroxetine or DMP696 (figure 6C).

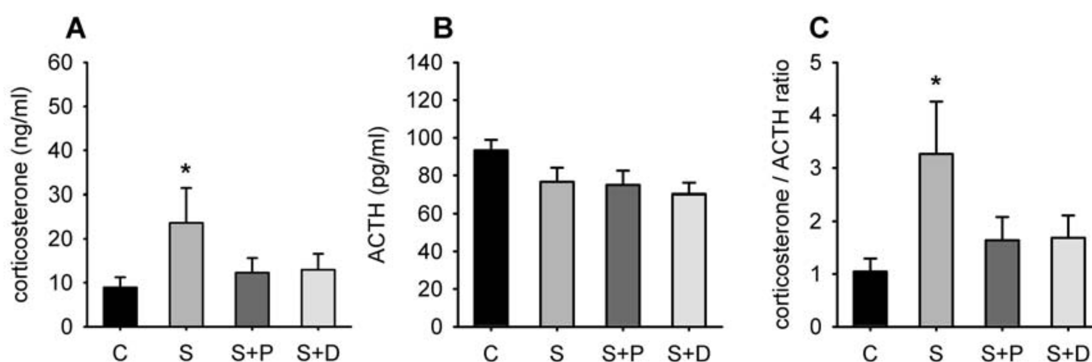

Figure 6: Influence of DMP696 or paroxetine treatment during chronic social stress exposure on morning corticosterone (A) and ACTH (B) as well as corticosterone/ACTH ratio (C) after one week of recovery. Control = C; chronic stress = S; chronic stress + paroxetine = S+P; chronic stress + DMP696 = S+D. N=12 per group. * significant from control group, $p<0.05$

We also analyzed basal gene expression of MR and GR mRNA in the hippocampus (figure 7). For MR, ANOVA revealed a significant condition effect (CA1: $F(3,34) = 5.236$ ($p<0.005$); CA3: $F(3,34) = 4.485$ ($p<0.01$); DG: $F(3,34) = 5.922$ ($p<0.003$)). The mRNA expression of MR in chronic stress animals was significantly decreased in the CA2 area compared to control animals. Paroxetine treatment reversed this effect in the CA2 area, and even significantly increased MR expression compared to control animals in the CA3 and dentate gyrus regions. DMP696 treatment had no effect on MR expression. For GR, ANOVA revealed a significant effect in the CA1 region ($F(3,29) = 4.186$ ($p<0.015$)). Chronic stress significantly decreased GR expression in the CA1 region. This effect was prevented by DMP696 treatment, while paroxetine treatment had no effect on GR expression in the chronic stress animals.

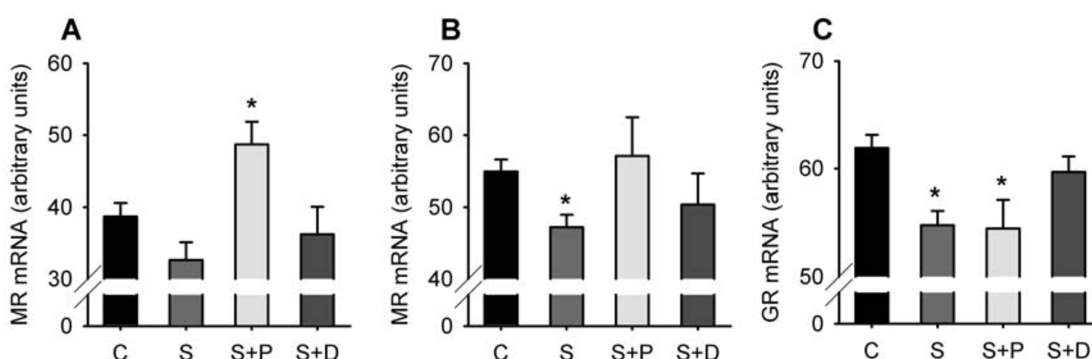

Figure 7: Influence of DMP696 or paroxetine treatment during chronic social stress exposure on long-term changes in MR mRNA expression in the CA1 (A), MR mRNA expression in the CA2 (B) and GR mRNA

expression in the CA1 (C). Control = C; chronic stress = S; chronic stress + paroxetine = S+P; chronic stress + DMP696 = S+D. N=12 per group. * significant from control group, $p<0.05$

Behavioral data

Based on the results obtained in experiment 1 we tested the animals according to anxiety-like behavior and locomotor activity.

We recorded the locomotor activity of the different groups in an open field test. For the locomotor adaptation in the first 5 minutes, ANOVA revealed a main effect of condition ($F(3,47) = 3.852$ ($p<0.016$)). Chronic stress resulted in a significant decrease of locomotor adaptation compared to control animals (figure 8A). This effect was reversed by paroxetine and DMP696 treatment. Basal locomotor activity during the last 20 minutes of open field exposure did not differ between the groups.

In the novelty-induced suppression of feeding test (figure 8B), animals which were chronically stressed showed a longer latency to consume the snack in the open field arena compared to control, DMP696 and paroxetine treated animals. However, due to a large variation in the chronic stress group, this effect was not significant (ANOVA: ($F(3,44) = 1.362$ ($p = 0.268$)).

In the elevated plus maze test ANOVA revealed a main effect of condition for the time spent in the open arms ($F(3,41) = 3.596$ ($p = 0.022$)). Chronic stress animals spent significantly less percent of time in the open arms of the plus maze and entered the open arms less often compared to controls (figure 8C-D). These effects were reversed by DMP696 treatment, but not by paroxetine treatment. Further, there was a significant effect in the number of head dips ($F(3,41) = 3.204$ ($p = 0.033$)). Chronic stress animals performed significantly less head dips compared to controls. Paroxetine or DMP696 treatment did not affect this parameter. There was no difference in the number of total arm entries between the groups, indicating similar levels of locomotor activity ($F(3,43) = 1.942$ ($p = 0.138$)).

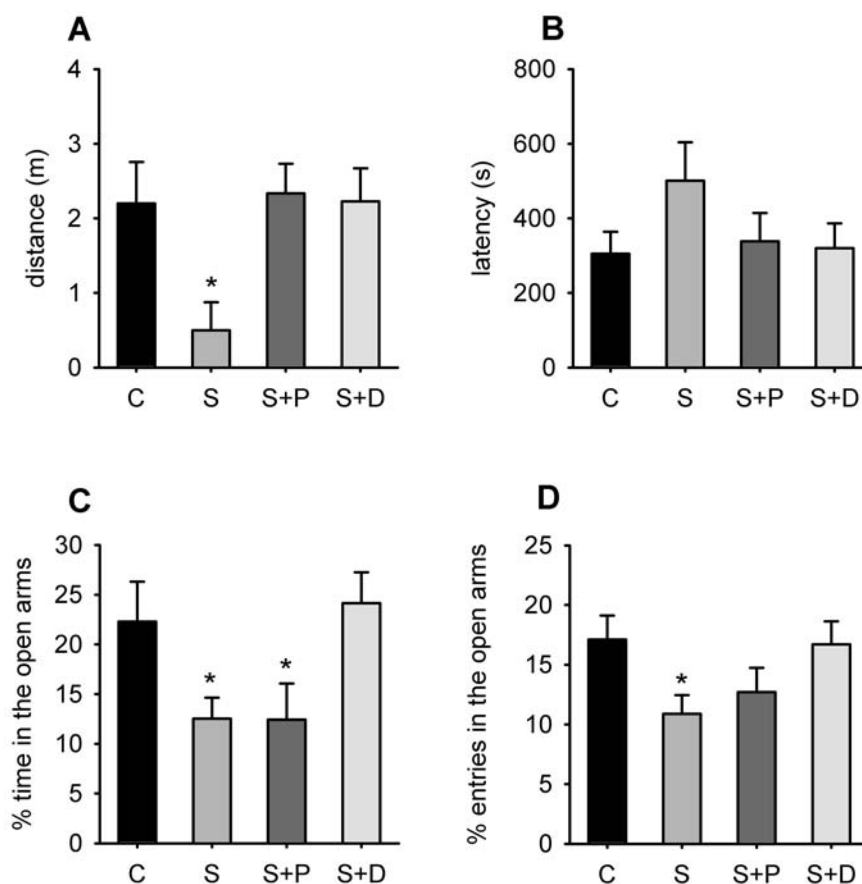

Figure 8: Influence of DMP696 or paroxetine treatment during chronic social stress exposure on long-term changes in anxiety-related behavior. Open field: Treatment effects on locomotor adaptation during the first 5 minutes (A). Novelty-induced Suppression of Feeding: Treatment effects on the time till initiation of almond consumption (B). Plus Maze: Treatment effects on time spent in the open arms (C) and number of entries to the open arms (D). Control = C; chronic stress = S; chronic stress + paroxetine = S+P; chronic stress + DMP696 = S+D. N=12 per group. * significant from control group, $p<0.05$

Discussion

In the present study we have characterized a novel chronic stress paradigm, which is based on social stress between group-housed male mice during adolescence. The stressor consists of an unpredictable social environment, which is constantly present during the stress procedure and unavoidable for the individuals. This paradigm was developed for three specific reasons. First, we aimed to create an animal paradigm of chronic social stress, where stress is actually chronic, unavoidable, non-adaptive and relevant to the clinical situation of stress-related pathophysiology. Second, the effects of chronic stress exposure should be evident and persistent even when the stressor is discontinued. Third, the paradigm should be applied during a time window of high vulnerability. Finally, the paradigm should be easily applicable without special equipment for a large number of

animals, in order to facilitate drug screening or studies investigating genetic and individual differences in the response to chronic social stress. Ideally, an animal model for a human clinical condition should display certain symptoms characteristic for the disease (face validity), the model should display reduced symptoms when treated with a clinically efficacious drug (predictive validity) and, finally, the neurobiological mechanisms underlying the symptomatology as well as the psychological causes should be identical (construct validity).

We have demonstrated that the application of this procedure indeed results in chronic stress for the animals. Most importantly, a variety of effects of this stress procedure are persistent, even when the stressor is discontinued and the animals are single housed under resting conditions. The measurement of long-term alterations of neuroendocrine and behavioral parameters due to chronic stress instead of the immediate effects of chronic stress is crucial, as human pathologies often develop with a latency to the experienced stress exposures (e.g. childhood trauma). Therefore we did not focus on the acute effects of chronic stress exposure, which often represent non-pathological adjustments to the stressful situation. So far there are only few reports in the literature that report long-term consequences of acute or chronic stress (Ahima et al., 1998). Our data showed that after 7 days of rest after the stress procedure previously chronically stressed animals display a lasting sensitization of adrenal activity. Morning ACTH levels of chronic stress animals were lower compared to controls, while the corticosterone production from the markedly enlarged adrenals was enhanced. Thus, lower ACTH concentrations are capable to induce a higher basal corticosterone secretion. In addition, these animals develop a flattened circadian corticosterone rhythm compared to non-stressed controls, a phenomenon which is also observed in depressed patients (Deuschle et al., 1997; Weber et al., 2000). The changes in adrenal and thymus weights also indicate a lasting adaptation of the HPA axis. These data are in concordance with the early work of LeMoal and Henry, who also found increased adrenals, thymus diminution and increased corticosterone secretion (Henry and Stephens, 1977; Klein et al., 1992).

In contrast to chronic stress paradigms during adulthood, we did not detect a decrease in body weight in the stressed animals, even though the changes in corticosterone secretion, adrenal and thymus weight in our paradigm were robust. There are a few potential explanations for this unexpected result. First, it is possible that body weight regulation differs between adult and juvenile mice. Thus, growth factors may be less influenced by differences in corticosterone secretion during adolescence. Alternatively, adolescent mice

may be able to compensate body weight loss more easily than adult mice. Thus, chronic social stress is not always accompanied by a decelerated gain in body weight.

The peripheral neuroendocrine data indicated that chronic stress exposure might have resulted in a permanent alteration of central stress regulation. Indeed we found a marked decrease of GR and MR expression in different sub regions of the hippocampus. These findings are in line with previous reports demonstrating a down-regulation of these steroid receptors in the hippocampus following chronic stress in other species (Herman and Spencer, 1998; Meyer et al., 2001; Bartolomucci et al., 2003a). In contrast to other reports, we did not find alterations in the expression of CRH or AVP in the chronically stressed animals (Ader, 1969). However, as these effects of altered CRH or AVP gene expression have always been observed directly at the end of a chronic stress procedure, it is quite likely that they have normalized in the absence of a stressor and do not persist after the stressor is discontinued. Alternatively, due to an altered negative feedback, differences of CRH and AVP expression may only be observed under conditions of an activated HPA axis. Thus, our data demonstrate a long-term alteration of the central and peripheral HPA function as a consequence of prolonged social stress exposure.

As chronic stress in humans is regarded as a risk factor for developing a number of psychopathological symptoms, among them anxiety and depression, we have examined the behavior of chronically stressed animals 7-14 days following the termination of the stress procedure. Chronic stress exposure permanently altered anxiety-related behavior compared to controls. Previously stressed animals showed a decreased locomotor adaptation in a novel environment compared to control mice, indicating an increased anxiety in the novel situation. Furthermore, chronic stress exposure resulted in a more anxious phenotype in the plus maze and novelty-induced suppression of feeding paradigm. Both tests have been intensively validated to reliably measure anxiety in rodents (Rex et al., 1998; Rodgers et al., 2002; Merali et al., 2003; Plamondon and Khan, 2005). Thus, certain aspects of anxiety are persistently affected by previous chronic stress exposure as a behavioral adaptation to the environmental demands.

Besides the increase in anxiety-like behavior, chronically stressed animals displayed other symptoms of stress exposure, such as decreased fur quality (Ducottet and Belzung, 2005). This deterioration of fur state can be explained by a decreased grooming behavior which represents a form of personal neglect and decreased grooming motivation.

In the second part of this study, we investigated whether the long-term effects of chronic social stress can be prevented by antidepressant treatment during stress exposure. For this

experiment we chose two drugs with different mechanisms of action, a selective serotonin reuptake inhibitor (paroxetine) and a CRH receptor 1 antagonist (DMP696). While the former is a well known and widely used antidepressant, the latter represents a new class of putative antidepressants, aimed directly at normalizing a central CRH hyper-drive of the HPA axis (Keck and Holsboer, 2001). Both compounds were able to normalize some of the long-term effects of chronic stress exposure on HPA system function and behavior. This finding is especially intriguing, as both drugs have completely different sites and mechanisms of action. As DMP696 inhibits both the peripheral and central CRHR1 system, lasting chronic stress effects mediated by elevated peripheral corticosterone concentrations or an enhanced activity of central CRH systems should be blocked by the drug. Accordingly, DMP696 treatment during stress exposure prevented peripheral alterations as adrenal enlargement, altered corticosterone/ACTH ratio and enhanced morning corticosterone levels. Interestingly, paroxetine treatment also normalized these peripheral HPA activity markers (Keck et al., 2003). In addition, both drugs were at least partly able to reverse the increased anxiety phenotype observed in chronic stress animals. Thus, even though both drugs act via different neurotransmitter pathways, their effects on stress hormone regulation are similar. These data further support earlier data relating an aberrant stress hormone regulation to causality of depression and suggesting that a normalization of the HPA system might be the final step necessary for stable remission of the disease (Holsboer, 1999; Holsboer, 2000). These findings are also in line with previous reports, suggesting the use of CRHR1 antagonists as novel antidepressant therapy (Zobel et al., 2000).

The different mechanism of action of both drugs becomes apparent when studying the central effects of paroxetine and DMP696. In concordance with previous findings, there is a significant decrease in MR and GR mRNA expression in the hippocampi of chronically stressed animals. Interestingly, paroxetine treatment affected mainly the expression of MR, while DMP696 treatment selectively prevented the decrease in GR expression. In non-stressed mice, acute CRHR1 antagonist treatment has already been shown to influence both MR and GR expression (Post et al., 2005). In addition, a number of studies indicated increased MR expression after chronic SSRI treatment, with no or little effect on GR expression (Lopez et al., 1998; Yau et al., 2002; Pariante et al., 2004). Thus, both drugs seem to influence peripheral HPA function by different central pathways.

A very interesting aspect of the persisting effects of chronic social stress in rodents and humans are individual differences. Some individuals seem to be highly vulnerable to

chronic stress exposure, while others are largely unaffected. The variability of our data is a strong indication for individual differences in stress resistance. As our chronic social stress model is ideally suited for the use of large experimental groups, the comparison of vulnerable and resistant phenotypes within a chronic stress group should provide valuable information on the interaction between genes and environment.

In summary, our novel chronic stress paradigm results in persistent alterations of HPA axis function and behavior. The neuroendocrine and behavioral consequences of chronic social stress can be partly prevented by treatment with a CRHR1 antagonist or by treatment with the SSRI paroxetine. Therefore, this stress paradigm fulfills the requirements of a reliable and valid animal model mimicking the human condition of stress-related diseases with respect to its etiology, symptomatology, treatment and biological basis. By applying this paradigm it will now be possible to investigate the interaction between genetic susceptibility and environmental factors, i.e. to what extent the long-term effects of chronic stress are dependent on a specific genetic background.

2. Long-term behavioral and neuroendocrine alterations following chronic social stress during adolescence: implications for stress-related disorders

Chronic stress and the resulting characteristic dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis clearly enhance vulnerability to a variety of diseases, including both affective and anxiety disorders (Chrousos and Gold, 1992; Anisman and Zacharko, 1992; Holsboer, 1999; McEwen, 2000; Amat et al., 2005). Chronically elevated cortisol levels are known to play an important role in the development of major depression in humans (Holsboer, 1999) and a high percentage of patients suffering from major depression are characterized by a hyperactive HPA axis (Holsboer and Barden, 1996; Rybakowski and Twardowska, 1999; Holsboer, 2000).

The mechanisms that lead to depression-associated HPA hyperactivity, including increased central corticotropin-releasing-hormone (CRH) levels (Raadsheer et al., 1994; Raadsheer et al., 1995), are still not completely understood. Genetic and experience-related factors may interact to induce manifold changes in corticosteroid receptor signaling, finally resulting in hypersecretion of both CRH and vasopressin (for review see: de Kloet et al., 1998). Several genetic susceptibility markers have recently been identified, for example a specific single nucleotide polymorphism in the FKBP5 gene, a co-chaperone modulating glucocorticoid receptor function, which is associated with an increased recurrence of depressive episodes (Pezawas et al., 2005; Ising and Holsboer, 2006). Besides genetic risk factors, increasing evidence shows an important role of environmental factors or stressful life events for the development of affective disorders in adulthood (Paykel et al., 1969; Coplan et al., 1996). Thus, both genetic predisposition as well as life experience seem to contribute to one's susceptibility to stress and acute life events, and the individual risk of developing depression. Moreover, normalization of the HPA system has been shown to be a prerequisite for stable remission of the disease (Zobel et al., 1999; Holsboer, 2000), which has been shown to occur during successful antidepressant treatment (Barden et al., 1995), again supporting the importance of the stress hormone system in the development and maintenance of affective disorders.

As experimental variation of early environment and stress exposure cannot be performed in humans for obvious reasons, preclinical studies using animal models are indispensable to improve our understanding of the consequences of chronic stress on stress responses later in life. Most of the effects described so far in the various existing stress models are limited to acute effects, observed only shortly after termination of the different stressors. In many

of these paradigms acute alterations of neuroendocrine parameters, like elevated CRH and arginine-vasopressin (AVP) expression levels, can be detected. Also, behavioral alterations could be observed in stressed animals displaying increased depression-like and anxiety-related behavior (Ottenweller et al., 1989; Aguilera, 1994; Gregus et al., 2005; Willner, 2005). However, the data on long-term effects of chronic stress are very limited (Tsankova et al., 2006), which are a crucial factor with regard to human pathology and disease development according to the stress-diathesis model (Morley, 1983). In addition, as the majority of stressful stimuli that enhance the risk for psychiatric disorders in humans are of a social nature, the etiology of human stress-associated disorders has to be taken into account.

Trying to overcome the limitations of the existing models, we recently developed a novel mouse paradigm for chronic social stress during adolescence (Schmidt et al., 2007), which is based on subjecting the animals continuously for seven weeks to an unstable social environment. Social stressors are likely to have a high impact during adolescence and early adulthood, which is a period of life that is highly adaptive and during which substantial remodeling occurs in areas involved in emotional and learning processes (Avital and Richter-Levin, 2005; Tsoory et al., 2007). We demonstrated significant effects of this chronic stress paradigm with respect to physiological, neuroendocrine and behavioral parameters after the stressor was discontinued for at least seven days (Schmidt et al., 2007). In addition, many of the adverse consequences evoked by the chronic stress could be prevented by simultaneous antidepressant treatment. Our data indicate that this paradigm closely meets the criteria of face, construct and predictive validity for chronic stress in humans (Willner, 1984; Geyer and Markou, 1995).

One of the central questions with regard to chronic stress exposure is which of the acute effects of the stress experience persist and in which way this early experience might enhance disease vulnerability. To address this question, we investigated the persistent effects of chronic social stress exposure 12 months after the cessation of the stressor in comparison to the observed acute effects. Our current data identify physiological, functional and behavioral parameters that are persistently altered or affected by chronic stress exposure throughout lifespan, thereby possibly altering the vulnerability of these animals for disease.

Experimental design

Experiment 1: immediate stress effects

For the acute effects of chronic social stress a total of 64 animals (32 control and 32 chronic stress animals) were used. A subgroup of 16 control and 16 chronic stress animals were used for neuroendocrine testing four days after the last change in group composition. Evening blood samples were taken between 16.00h and 18.00h by tail cut (Fluttert et al., 2000}. On the next morning, the animals were sacrificed under basal conditions (see: Sampling procedure). The remaining animals were tested in the following behavioral tests: elevated plus maze, novelty-induced suppression of feeding and open field. Body weight was determined every two weeks prior to cage change.

Experiment 2: persistent stress effects

For the investigation of persisting effects of chronic stress in aged animals a total of 44 animals (22 control and 22 chronic stress animals) were used. A subgroup of 10 control and 10 chronic stress animals were used for neuroendocrine testing at the age of 15 months, i.e. 12 months after termination of the stress and group housing. The animals were sacrificed in the morning under basal conditions. The remaining animals were tested in the following behavioral tests: elevated plus maze, novelty-induced suppression of feeding and open field test.

Results

Experiment 1

Physiological data

There were no differences between the groups in mean body weight during the experiment (t-test, $p=0.366$ at the end of the stress period). Adrenal and thymus glands of 16 control and 16 chronic stress animals, sacrificed four days after the last change in cage composition, were dissected and weighed (figure 9). The relative weight of the adrenal glands in chronically stressed animals was significantly increased (t-test, $p<0.05$), while relative thymus weight was significantly decreased (t-test, $p<0.05$).

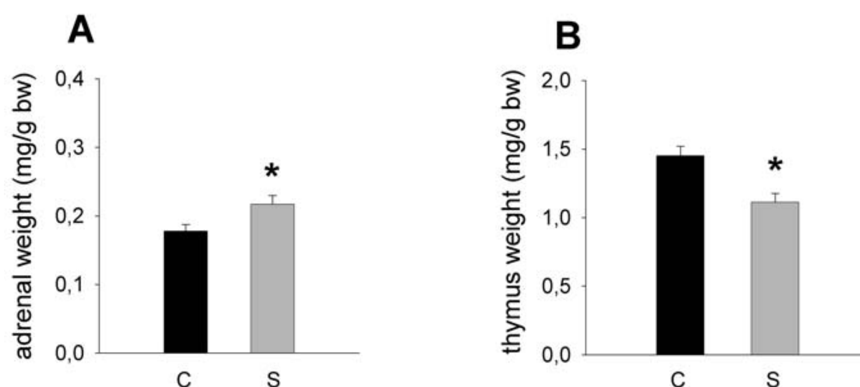

Figure 9: Immediate effects of chronic stress on adrenal weight (A) and thymus weight (B). control = C; chronic stress = S; N = 16 per group. * significant from control group, p<0.05

Neuroendocrine data

Four days following the last cage member rotation, basal evening and morning corticosterone as well as morning ACTH plasma levels were measured. For corticosterone, ANOVA with repeated measures (time as within factor, treatment as between factor) revealed a significant effect of time (F (1,19) = 39.450 (p<0.001)) and a significant interaction between time and condition (corticosterone: (F (1,19) = 14.908 (p<0.01)). Chronic stress animals had significantly higher corticosterone levels in the morning (p<0.05) and lower levels in the evening (p<0.05) (figure 10A), resulting in a flattening of the circadian rhythm (figure 10B). For ACTH no significant differences in basal morning levels could be observed (t-test, p=0.208) (figure 10C). Further, the morning corticosterone/ACTH ratio in chronic stress animals was significantly higher compared to controls (t-test, p<0.05) (figure 10D).

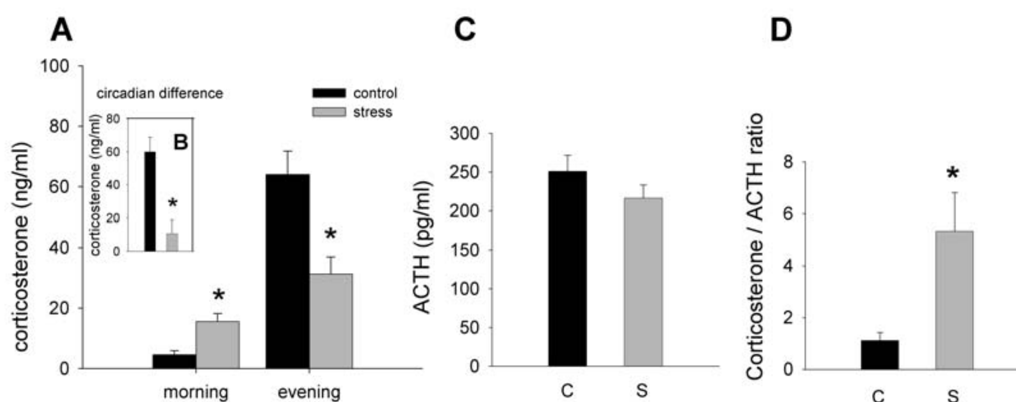

Figure 10: Immediate effects of chronic stress exposure during adolescence on HPA axis function. (A) Morning and evening corticosterone levels after seven weeks of chronic stress. (B) Circadian difference of corticosterone levels. (C) Basal ACTH levels. (D) Corticosterone / ACTH ratio after chronic stress. control = C; chronic stress = S; N = 16 per group. * significant from control group, p<0.05

We also analyzed basal gene expression of CRH and AVP mRNA in the PVN as well as MR and GR mRNA expression in the hippocampus (figure 11). The expression of AVP in the PVN was significantly higher in the chronic stress group (t-test, $p<0.05$). No alteration in CRH expression was observed between the two groups (t-test, $p=0.474$).
For the MR mRNA levels we found a significant decrease in the CA1, CA2, CA3 area and the dentate gyrus of the hippocampus in chronically stressed animals (t-test, $p<0.05$). GR mRNA was significantly decreased in the CA1 region (t-test, $p<0.05$).

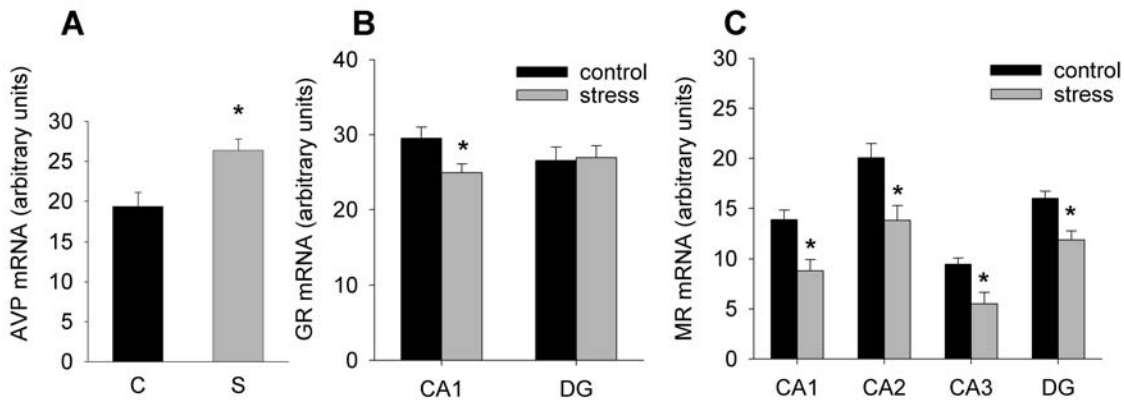

Figure 11: Expression levels of AVP mRNA in the parvocellular part of the PVN (A), GR mRNA in the CA1 region and the dentate gyrus of the hippocampus (B) and MR mRNA in the CA1, CA2, CA3 region and the dentate gyrus of the hippocampus (C). control = C; chronic stress = S; N = 12 per group. * significant from control group, $p<0.05$

Behavioral data

To asses alterations in behavior due to chronic stress exposure we tested the animals in anxiety-related behavioral tests. In the elevated plus maze (figure 12A), control animals entered the open arms significantly more often (t-test, $p<0.05$) and tended to spent more time in the open arms (t-test, $p<0.1$) compared to animals from the chronic stress group. The number of closed arm entries was not different between the groups, indicating a similar locomotor activity in this behavioral paradigm. Also the number of head dips, which is one of the best indicators for anxiety in the elevated plus maze (Fernandez, 1997), was significantly higher in controls (figure 12B) (t-test, $p<0.05$).
Chronically stressed animals also displayed an enhanced anxiety-related behavior in the novelty-induced suppression of feeding paradigm (figure 12C), where control animals initiated the almond consumption significantly faster compared to previously stressed animals (t-test, $p<0.05$).
In the open field, stressed animal tended to travel less in the first two minutes of the test, although the differences were not statistically significant ($F(4,30) = 2.560$ ($p<0.1$)) (figure

12D). The pattern of locomotion did not differ between both groups in the last three minutes of the test.

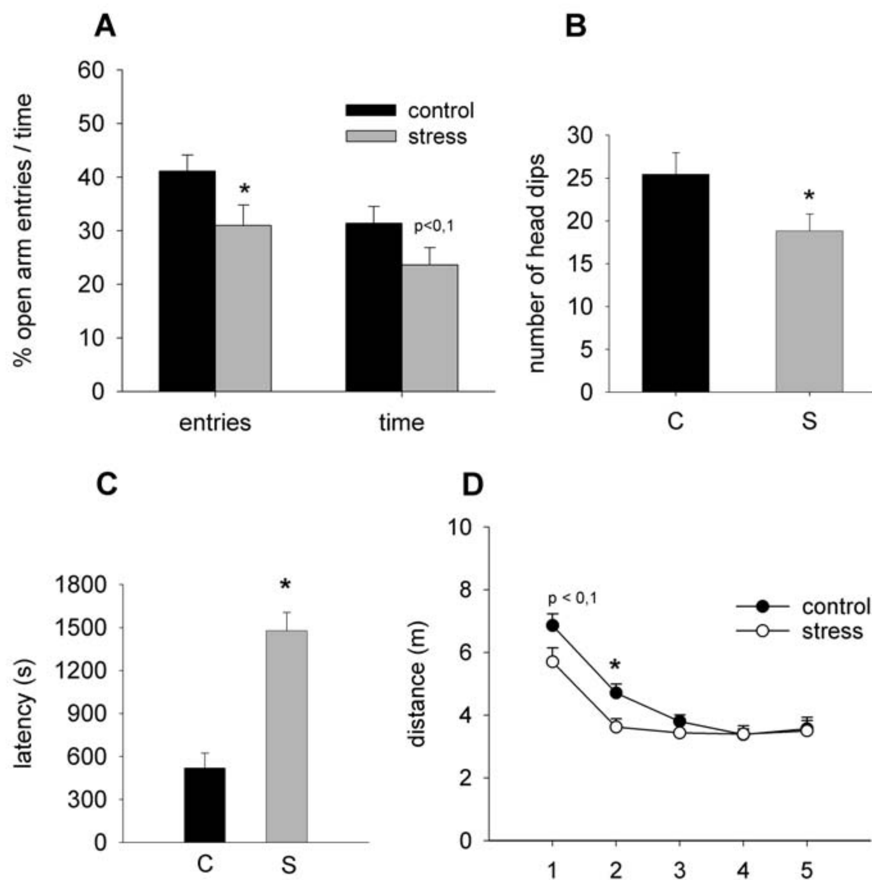

Figure 12: Elevated plus maze: immediate effects of chronic stress on (A) the number of entries into the open arms and (B) on the number of head dips. (C) Novelty-induced suppression of feeding: effects of chronic stress on the time till initiation of consumption of a palatable snack. (D) Open field: effects of chronic stress on the locomotor activity of the animals. control = C; chronic stress = S; N = 16 per group. * significant from control group, $p<0.05$

Experiment 2

Physiological data

There were no differences between the groups in mean body weight during any time of the experiment (t-test, $p=0.347$ at the age of 15 months). The adrenal glands of 10 control and 10 chronic stress animals sacrificed at the age of 15 months were dissected and weighed. The relative weight of the adrenal glands did not differ between the two groups anymore (t-test, $p=0.521$). Due to the natural shrinkage of the thymus gland over lifetime its weight could not be investigated in the aged mice.

Neuroendocrine data

Basal morning corticosterone levels were determined, revealing no significant difference between the two groups (t-test, $p=0.592$). Gene expression analyses of CRH and AVP mRNA in the PVN as well as GR and MR mRNA expression in the hippocampus were performed (figure 13). The expression levels of AVP (figure 13A) and CRH mRNA were not significantly different between both groups of aged mice (t-tests, $p=0.115$ for AVP and $p= 0.35$ for CRH, respectively).

For GR mRNA, a decrease could be detected in the CA1 region and the dentate gyrus in the stress group, but this effect failed to reach statistical significance (t-test, $p<0.1$) (figure 13B). For MR mRNA levels we found a significant decrease in the CA3 area and the dentate gyrus of the hippocampus in chronically stressed animals (t-test, $p<0.05$) (figure 13C).

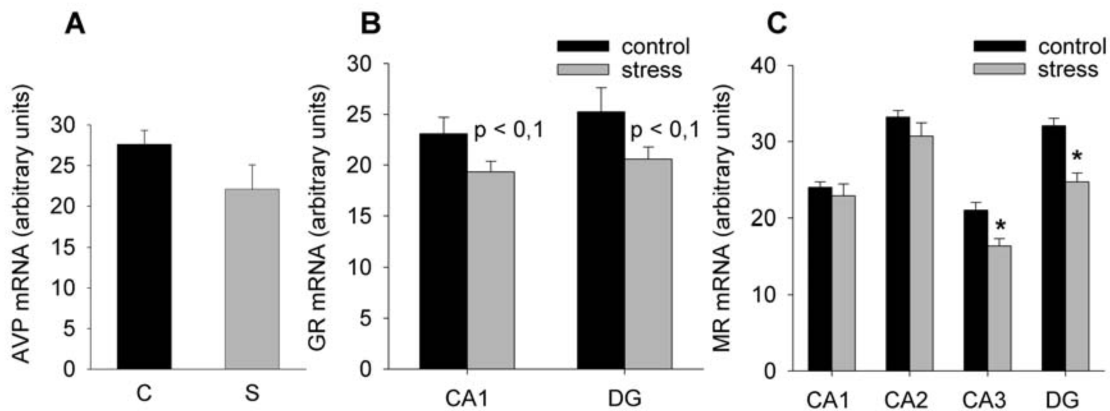

Figure 13: Expression levels of AVP mRNA in the parvocellular part of the PVN (A), GR mRNA in the CA1 region and the dentate gyrus of the hippocampus (B) and MR mRNA in the CA1, CA2, CA3 region and the dentate gyrus of the hippocampus (C) in aged animals. control = C; chronic stress = S; N = 10 per group. * significant from control group, $p<0.05$

Behavioral data

For the behavioral characterization, locomotor activity and performance in anxiety-related tests were assessed. In the elevated plus maze, control animals did not differ from stress animals in terms of open arm entries (t-test, $p<0.1$) (figure 14A), time spent in the open arms, the number of closed arm entries and the total distance traveled. However, the number of head dips was still significantly higher in controls compared to previously stressed animals (figure 14B) (t-test, $p< 0.05$).

In the novelty-induced suppression of feeding paradigm no persistent effects could be detected (figure 14C). Stress animals tended to feed the snack later than controls, but due to a high individual variation within each group this difference was not significant.

Chronically stressed and control animals initiated the snack consumption in a similar time range.

In the open field, the locomotor adaptation of the animals was investigated. We found an overall effect between both groups ($F(4,21) = 3.433$ ($p<0.05$)) with the previously stressed animals displaying decreased locomotor adaptation compared to controls, predominantly in minute three and four of the test ($p<0.05$) (figure 14D).

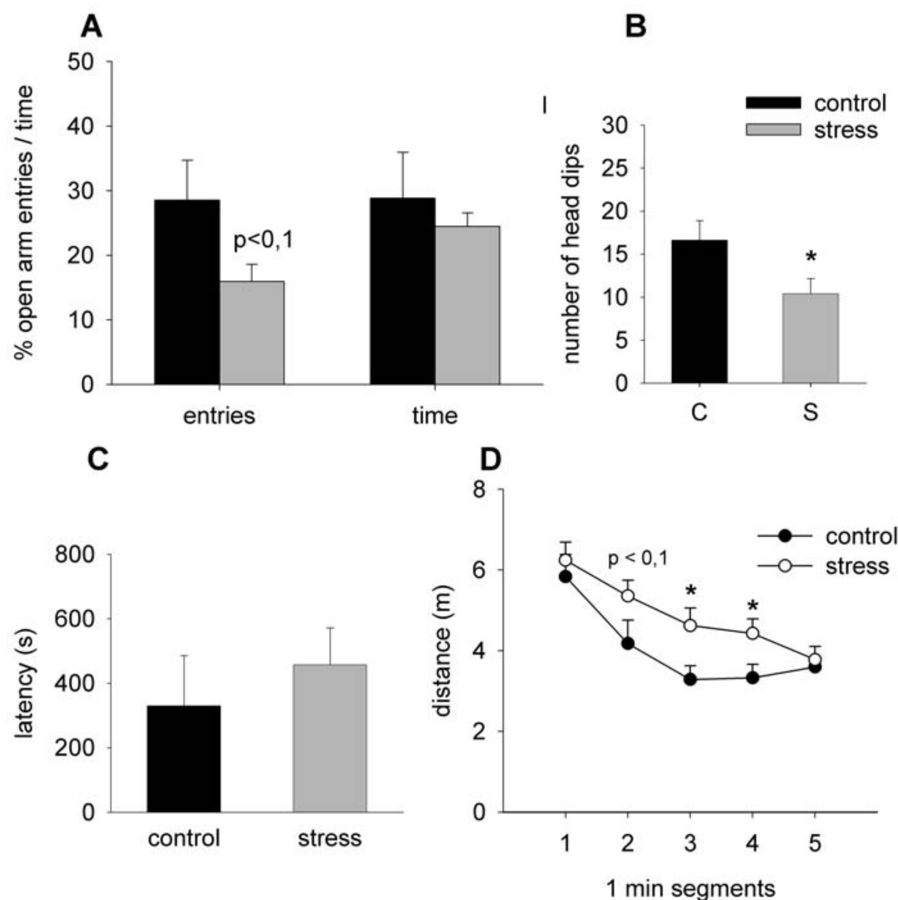

Figure 14: Elevated plus maze: persistent effects of chronic stress on the number of entries into the open arms (A) and on the number of head dips (B). (C) Novelty-induced suppression of feeding: persistent effects of chronic stress on the time till initiation of consumption of a palatable snack. (D) Open field: persistent effects of chronic stress on the locomotor pattern of the animals. control = C; chronic stress = S; N = 10 per group. * significant from control group, $p<0.05$

Discussion

The aim of the present study was to characterize persistent neuroendocrine and behavioral effects of chronic social stress during adolescence obtained 12 months after cessation of the stressor. As a reference, and also to dissect acute stress effects from persistent ones, we also investigated the immediate effects of chronic stress obtained at the end of the chronic stress period. Our findings provide evidence that chronic stress exposure during a crucial developmental time period results in long-term, persistent effects on physiological and

behavioral parameters throughout life, which – if transferred to the human situation - may contribute to an enhanced vulnerability to stress-induced diseases, such as affective disorders (Kendler et al., 1999; Kendler et al., 2001).
We could demonstrate robust immediate effects of the chronic stress paradigm at the behavioral and neuroendocrine level, adding further evidence for the validity of this novel animal model for chronic stress research (Schmidt et al., 2007). At the end of the stress period chronically stressed animals displayed significantly higher basal morning and lower evening corticosterone levels, resulting in a flattened circadian rhythm compared to non-stressed controls. Interestingly, a shift or flattening of circadian stress hormone secretion is a phenomenon that is also observed in depressed patients (Deuschle et al., 1997; Weber et al., 2000). Both the increase in adrenal weights and the decrease in thymus weights are robust immediate effects after the chronic stress procedure, further indicating a chronic activation of the HPA axis during the stress phase. In addition to alterations in peripheral hormone secretion, we could detect concomitant changes in central stress parameters: We found a marked decrease of GR mRNA and MR mRNA expression in different sub regions of the hippocampus as well as a marked increase in AVP mRNA expression in the hypothalamic PVN. These findings are in line with previous reports demonstrating a down-regulation of hippocampal steroid receptors or an up-regulation of hypothalamic AVP following chronic stress procedures (Makino et al., 1995; Albeck et al., 1997; Herman and Spencer, 1998; Meyer et al., 2001; Bartolomucci et al., 2003a). As chronic stress is regarded a key risk factor to develop a number of psychopathological symptoms in humans including anxiety and depression-like symptomatology (Kendler et al., 1999; Kendler et al., 2001), we investigated the behavior of chronically stressed animals in relevant behavioral paradigms. Generally, chronic stress exposure increased anxiety-related behavior compared to controls. Chronically stressed animals displayed a more anxious phenotype immediately after stress termination in the novelty-induced suppression of feeding paradigm and the elevated plus maze,. Both tests have been validated to reliably measure anxiety in rodents (Rex et al., 1998; Rodgers et al., 2002; Merali et al., 2003). As expected, the immediate effects in this paradigm are more pronounced compared to the effects obtained seven to ten days after stress cessation as described earlier (Schmidt et al., 2007). For instance, we now detected a significant increase in hypothalamic AVP mRNA expression, which seems to represent a more acute adaptation to the stressful situation, as it has been found to be normalized again seven days after the stress exposure.

With respect to the human situation, where stressful life events have been shown to significantly increase the risk for the development of depressive disorders later in life, the question of whether social stress during adolescence is able to induce long-term alterations of HPA system regulation is of particular importance. To address this question, we investigated physiological, neuroendocrine and behavioral parameters in aged, 15-month-old animals (i.e. 12 months after the termination of the stress procedure), in order to determine potential persistent effects of chronic social stress during adolescence. As expected, most of the pronounced acute effects of the stress exposure had normalized and the two experimental groups showed a similar phenotype in many parameters. However, even after 12 months of living in a stress-free environment, animals previously subjected to chronic stress during adolescence markedly differed in some specific aspects of behavior and physiology from non-stressed controls. We still observed a remarkable decrease of MR mRNA expression in the hippocampus of previously stressed animals, closely resembling the situation directly at the end of the stress paradigm 12 months earlier. Further, behavioral parameters indicating aspects of anxiety-related behavior, such as head dips on the elevated plus maze (Fernandez, 1997) or locomotor adaptation in a novel environment were found to be significantly different between both groups. These data indicate that the profound challenge of social stress exposure during adolescence and early adulthood had persistent effects on the behavior of the animals even at an old age.

Given their potential to modulate principal electrophysiological properties of limbic neurons, it becomes evident that glucocorticoids play important roles in modulating fear and anxiety-related behavior (for review: Korte, 2001). As the mechanisms by which corticosteroids exert their effects on behavior are often indirect by modulating particular sets of neurons or neurotransmitter systems, contrasting effects of corticosteroids on fear, anxiety and depressed mood have been described. Aspects of fear and anxiety are affected differentially by the occupation of the MR or GR at different phases of the stress response. What might be the impact of persistent mineralocorticoid receptor down-regulation for the pathophysiology of stress-related psychiatric disorders? The cumulative evidence makes a strong case implicating corticosteroid receptor dysfunction in the pathogenesis of affective disorders (Neigh and Nemeroff, 2006; Holsboer, 2000). In this context, the observation that antidepressant treatment in rodents increases levels of hippocampal MR (Reul et al., 1993) and that MR receptor antagonism is unfavorably influencing the response to antidepressants, point to an important role of central MR in mediating antidepressant response (Holsboer, 1999). Persistent down-regulation of hippocampal MR mRNA in our

paradigm 12 months after cessation of the social stress exposure could therefore represent such a stable, stress-induced individual vulnerability factor, which in humans may predispose to the development of affective disorders later in life (Brunson et al., 2001).

There are several potential explanations for the persistence of the chronic stress effects. Epigenetic modifications of DNA promoter regions have recently been shown to be modified by environmental influences, resulting in persistent changes of gene expression. Weaver and colleagues demonstrated that high licking and grooming behavior of rat mothers reduced the DNA methylation of a specific region of the GR promoter, which resulted in an altered GR expression and subsequently in physiological and behavioral changes (Weaver et al., 2004). A similar mechanism of action could also be postulated for the observed long-term effects of chronic stress exposure during adolescence, although there is so far little direct evidence for epigenetic programming in adult animals. Alternatively, chronic stress exposure might have resulted in structural changes in specific brain regions, possibly affecting neuronal number, dendritic branching or spine density. All of these structural parameters have previously been shown to be affected by high levels of glucocorticoids and could be responsible for long-term changes in behavior or function (Magarinos et al., 1988; Kleen et al., 2006).

The observation that consequences of chronic stress during adolescence persist to late adulthood in animals is also important with regard to human studies that relate early trauma or adverse experiences with long-term effects (Heim and Nemeroff, 2001). It is well documented, that trauma during early life including puberty, like physical or sexual abuse, or life events, enhance the individual risk to develop affective disorders like depression in adulthood (McCauley et al., 1997). Genetic predisposition combined with experiencing early adverse events in vulnerable phases of development may both shape the individuals' susceptibility to subsequent stressors and hence lead to manifestation of a disease.

In contrast to other animal models revealing persistent effects of early postnatal stress, such as maternal separation of the pups (Plotsky and Meaney, 1993), we are able to show for the first time relevant long-term consequences of social stress that the animals were exposed to during adolescence. Maternal separation is usually performed in the neonatal phase (day 2-15) during the stress-hyporesponsive period in which HPA system regulation is markedly different from stress hormone regulation in the adult animal (Levine, 2001). As in our model chronic stress takes place between days 28 and 81 of the animals' life (i.e. in adolescent / adult animals), our data might be of particular relevance for the human situation with respect to the pathophysiology of stress-related psychiatric disorders.

Epidemiological studies clearly show, that stress alone is not sufficient to result in the manifestation of a disease. In many of the neuroendocrine and behavioral parameters we assessed at an old age we observed a trend, which probably did not reach the level of significance due to a high variability in the group. One might speculate that only a sub-population of the previously stressed animals are largely and persistently affected by the stress exposure due to their specific genetic makeup, while other animals "recover" and are not at an enhanced risk for disease. As with the here applied chronic stress model it is feasible to subject a large number of animals to the stressful situation, future studies will have to focus on the individual differences between chronic stress animals in terms of the magnitude of persistent effects and their interaction with genetic vulnerability of the individuals. Furthermore, it will be interesting to study the possible pharmacological modification of persistent effects of chronic stress experiences.

In summary, the application of chronic social stress during adolescence exerted robust immediate effects on HPA axis activity, gene expression, and anxiety-related behavior. These data support our previous findings and point to the high face, construct and predictive validity of this novel animal model for chronic social stress (Schmidt et al., 2007). In addition, persistent effects of the chronic social stress could be detected in aged animals one year after stress termination. With respect to the postulated causal relationship between chronic stress and the development of human affective disorders, these data are the basis to study the underlying neurobiological mechanisms in detail. A better understanding of those pathophysiological mechanisms, in turn, will contribute to the development of novel, innovative, antidepressant drugs in the future.

3. Lasting consequences of chronic stress during adolescence on cognition

Cognitive dysfunction is known to play a pivotal role in a variety of diseases including major depression and anxiety disorders and there is evidence that cognitive impairment may in fact underlie many of the affective symptoms (Anisman and Matheson, 2005; Mathews and Mackintosh, 1998). On the other hand, late-onset depressive symptoms can often be a predrome of cognitive decline and represent early manifestations of memory disorders (Steffens et al., 2006; Charney et al., 2003). Sustained elevated glucocorticoid levels, resulting from a hyperactive HPA axis, have been hypothesized to represent a risk factor for both depressive disorders and cognitive impairment.

Unlike brief periods of stress, which are rather thought to enhance cognition (Lupien et al., 2007; McEwen and Sapolsky, 1995; McEwen, 1999; Shors et al., 1989; de Kloet et al., 1998), chronic stress may be very detrimental and constitutes a key risk factor for diseases that affect memory performance. Permanently increased circulating cortisol levels could be associated with impairment in various cognitive domains like attention, perception and memory as well as reduced hippocampal volume (Newcomer et al., 1999; Jameison and Dinan, 2001).

There are several animal models that try to relate the impact of stressors during different stages of life with cognitive decline in late adulthood or senescence. Brunson and colleagues could show that chronic early life stress (i.e. post natal day 2-9) resulted in selective, severe impairment in spatial and object recognition memory in middle-aged but not young adult rats. The process of cognitive decline therefore seems to be progressive, induced by the early aversive experience (Brunson et al., 2005). In adult rats, repeated social defeat affected neurophysiological aspects of hippocampal functioning reflected in LTP and LTD up to nine months following defeat (Van der Harst, 2003; Buwalda et al., 2005). Also exposure to social stress during adulthood has adverse effects on memory as shown by Bodnoff and colleagues, who stressed rats for six months from ten months of age on, resulting in impairments in spatial memory detected by using the Morris water maze task (Bodnoff et al., 1995). In adult mice, both learned helplessness or chronic mild stress have been shown to result in spatial memory impairment in the Morris water maze (Song et al., 2006), although no lasting effects of the stress procedures were described.

Another very important and often overlooked phase in life is the adolescent period. During this time of physical and sexual maturation, the brain is in a state of high plasticity, but also high vulnerability (McCauley et al., 1997). To study this critical life period, we have

recently developed a novel mouse paradigm for chronic social stress during adolescence and young adulthood, which closely resembles the human situation of social stress in terms of construct, face and predictive validity (Schmidt et al., 2007). Using this paradigm, which is based on the creation of a highly unstable social environment, we recently demonstrated long-lasting effects of stress exposure on neuroendocrine and behavioral parameters 12 months after cessation of the stressor (see chapter 3). However, while various persistent effects of this novel stress paradigm are already described, there are no data available on effects of cognition and memory.
In the current study, we investigated the long-term consequences of chronic social stress during adolescence on cognitive performance in 15-month-old animals. Further, we correlated the behavioral findings with electrophysiological and neurogenetic parameters involved in memory processes, in order to probe the molecular and physiological basis of the observed phenotype.

Experimental design

Experiment 1

In order to assess memory performance in aged mice, a total of 40 mice (20 chronically stressed and 20 control animals) were used. After the chronic stress period all animals were separated and since the kept in single housing. 12 months after cessation of the stressor, at the age of 15 months, all animals were subjected to cognitive testing. Half of the mice (10 control and 10 chronic stress animals) were tested in the Y-maze and the social discrimination task, the second half in the object recognition test and the Morris water maze. After testing, all animals were sacrificed under basal conditions and their brains were removed and kept frozen for gene expression analyses via in situ hybridization.

Experiment 2

In a subsequent experiment a second batch of mice was subjected to the chronic social stress paradigm and afterwards kept in single housing till the age of 15 months, analogously to experiment 1. The brains of 10 chronic stress and 10 control animals were dissected and one hemisphere was used immediately for electrophysiological recordings to assess potential alterations in hippocampal long-term potentiation (LTP). The other hemisphere was frozen and kept at -80 °C.

Results

Experiment 1

Behavioral data

To asses potential alterations in cognitive performance due to early chronic stress exposure, we tested the animals at an old age in relevant learning and memory tasks. In the social discrimination test, the animals of the control as well as of the stress group explored the novel female significantly longer compared to the familiar one, suggesting an intact social memory. No differences between both groups were detected at an intertrial interval of one hour (t-test, p=0.758). The same holds true for the object recognition test, where both groups explored the novel object significantly more often and longer compared to the known object but did not differ from each other in exploration times (t-test, p=0.365), indicating an intact object recognition memory. In the Y-maze, at an intertrial interval of one hour the animals of the stress group explored the additional plus arm significantly less often compared to controls (t-test, p<0.05), suggesting an impairment in the hippocampus-dependent spatial memory (figure 15A). This finding could be confirmed by the results of the Morris water maze. Both groups were able to reduce their needed time to locate the submerged platform on successive trials on the first two test days. However, on day three, the animals of the stress group required significantly longer distances and latencies to escape onto the submerged platform (overall condition effect revealed by ANOVA with repeated measures: (F(2, 57) = 3.207 (p<0.05)), post-hoc significant on day 3 for all trials) (figure 15B).

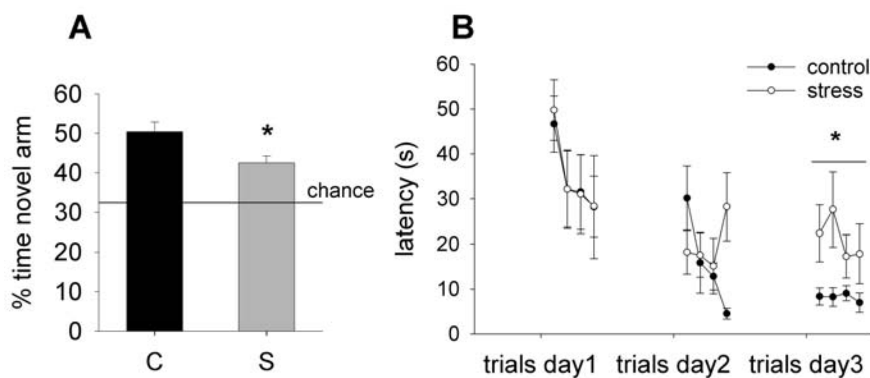

Figure 15: (A) Y-maze: long-term effects of chronic stress on the time exploring the additional plus arm. (B) Morris water maze: long-term effects of chronic stress on the time spent before locating a submerged platform. control = C; chronic stress = S; N = 10 per group. * significant from control group, p<0.05

Neurogenetic data

We analyzed basal gene expression of relevant neurotransmitter receptors involved in learning and memory in the hippocampus 12 months after stress termination. For the AMPA 1 receptor subunit GluR1 we could detect a significant increase in the GluR1 mRNA in the CA1, the CA3 and the dentate gyrus region of the hippocampus in the chronic stress group (t-test, p<0.05) (figure 16A). For NMDA1 mRNA no differences were found between both groups and for GABA A mRNA we could detect a significant decrease in the dentate gyrus (p<0.05) as well as a statistical trend in the CA1 region (p<0.06) (figure 16B). Furthermore, we determined the mRNA expression levels of the neurotrophine BDNF in the Ca1, CA3 and DG region of the hippocampus as well as in the cortex. In previously stressed animals a significant decrease of BDNF mRNA could be detected in all of these regions (t-test, p<0.05) (figure 17).

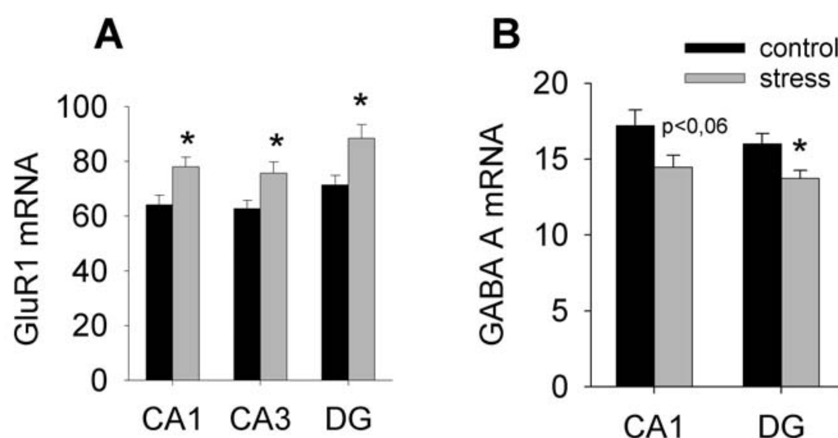

Figure 16: Long-term effects of chronic stress on neurogenetic data: mRNA expression of hippocampal (A) GluR1 and (B) GABA A receptor subunits. N = 12 per group. * significant from control group, p<0.05

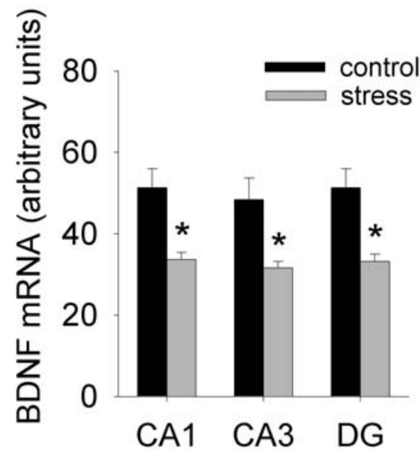

Figure 17: Long-term effects of chronic stress on BDNF mRNA expression levels in the hippocampus. N = 12 per group. * significant from control group, p<0.05

Experiment 2

Electrophysiology

To assess whether impairments in cognitive performance due to early chronic stress exposure coincided with alterations in synaptic plasticity in the CA1 region of the dorsal hippocampus, we studied long-term potentiation in the Schaffer collateral-commissural pathway in acute brain slices from control and stressed animals. After induction of LTP by high-frequency stimulation, slices of previously stressed mice showed a significantly weaker LTP than control littermates for at least 60 minutes after high-frequency stimulation (control: 194.3 ± 15.5%, n = 18; stressed: 146.4 ± 9.8%, n = 19; t-test, p<0.05) (figure 18).

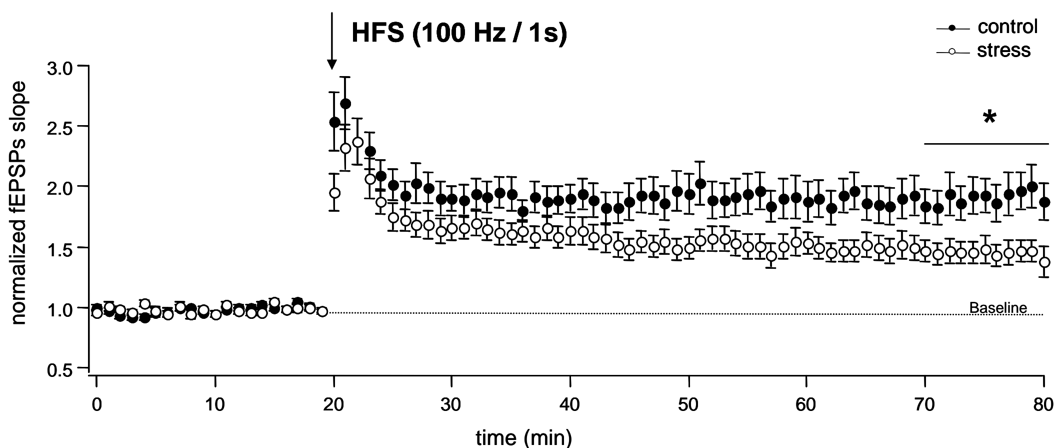

Figure 18: Long-term effects of chronic stress on long-term potentiation in the hippocampal CA1 area. N = 10 per group. * significant from control group, p<0,05

Western blots

Chronic stress might have induced adaptational changes within various neurotransmitter systems involved in learning and memory. Therefore, we analyzed the hippocampal region for the protein expression levels of the NMDA-receptor subunits NR2A and NR2B. Western blot analyzes revealed no difference in the protein expression levels of NR2A (t-test, p=0.885) (figure 19A), while the NR2B subunit seemed to be increased in previously stressed animals (figure 19B). However, due to large individual differences in the stress group this effect did not reach statistical significance (t-test, p<0.1).

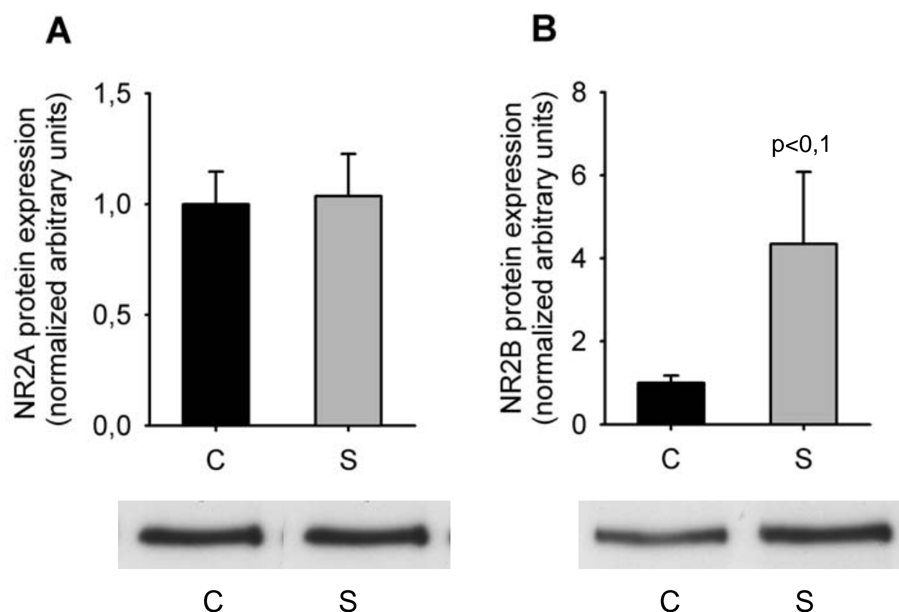

Figure 19: Long-term effects of chronic stress on neurogenetic data. Hippocampal protein levels of (A) NMDA receptor 2A and (B) NMDA receptor 2B. N = 10 per group. * significant from control group, p<0.05

Discussion

Our current data demonstrate for the first time effects of a chronic social stress exposure during adolescence on memory performance during aging. One year after cessation of the chronic social stress regimen, a substantial impairment of spatial memory could be detected in the previously stressed animals compared to controls. These findings were further supported by electrophysiological and neurogenetic data, indicating the hippocampal formation as main target for these long-term consequences of chronic social stress.

By applying various memory tests in 15-months old mice, only differing in their stress history during adolescence, we investigated learning and memory performance of different memory domains. These tests revealed that previously stressed mice displayed a memory deficit specific for spatial memory, while other memory domains such as social or object

recognition memory seem not to be differentially affected. As the hippocampal formation has previously been shown to be specifically involved in spatial memory processes (Squire, 1992; Squire and Zola-Morgan, 1991; Zola-Morgan et al., 1994; Nadel, 1991; Duva et al., 1997; Jarrard, 1993; O'Keefe and Nadel, 1978), we focused our further functional analysis on this structure. In fact, for memory performance in social or object recognition tasks key roles of non-hippocampal structures including the amygdala, the rhinal cortex and the prefrontal cortex have been implicated (Otto and Eichenbaum, 1992; Mishkin and Murray, 1994; Mumby and Pinel, 1994). Using electrophysiological recordings, we could detect reduced in vitro LTP in the hippocampal CA1 region of stressed animals 12 months after cessation of the stressor. Based on the suggested involvement of LTP in memory formation, these data imply a direct electrophysiological correlation with our behavioral data. This finding is in line with other studies that associated behavioral stress with an impairment in the induction of long-lasting activity-dependent changes in synaptic efficacy, such as LTP, later in life (Brunson et al., 2005; Artola et al., 2006; von Frijtag et al., 2000; Gerges et al., 2004; Diamond et al., 2004). Thus, it seems likely that stressful early life experience during adolescence lastingly affects hippocampal function.
There are at least two main possibilities of how these differences in hippocampal function can be mediated. First, it is possible that differences in transcription or post-transcriptional regulation of neurotransmitter systems involved in learning and memory processes are permanently altered. Second, long lasting structural changes of hippocampal dendritic branching or dendritic spine number or number of neurons could result in a different hippocampal function. To address the first possibility, we investigated expression levels of several neurotransmitter receptor subunits, which are known to play a key role in hippocampal learning and memory. Indeed we observed significant differences between previously stressed and control animals in hippocampal mRNA expression levels in terms of a GluR1 up-regulation and GABA down-regulation. Further we detected an increase in hippocampal NR2B protein levels in previously stressed animals, albeit due to a large individual variation in the stress group this increase was not statistically significant. As the animals of the chronic stress group had shown reduced in vitro LTP, based on literature reports from animal studies one would rather assume the converse regulation pattern. Particularly an over expression of NR2B is strongly associated with enhanced learning abilities in mice and rats (Tang et al., 1999). However, we should keep in mind that in those studies the animals used were younger in age which contrasts the present study, which was conducted with 15-month-old animals. This difference might be an important detail as it is

currently not known how a general state of over excitation resulting from a persistently increased expression of excitatory glutamate receptors and a reduced number of GABA receptors, potentially leading to increased intracellular calcium levels among other changes, impacts the CNS and possibly leads to a state of neuronal stress and finally excitotoxicity. Also in patients with moderate or severe cognitive impairment a reduction in GABA A mRNA had been demonstrated, which occurs probably very early in the progression of Alzheimer's disease (Rissman et al., 2004). In addition to the above mentioned receptors, downstream second messenger cascades regulating the activity of relevant kinases and phosphatases are highly involved in cognitive processes and alterations in their efficacy might also severely contribute to the observed reduction in in vitro LTP, independently from the receptor subunit expression patterns.

What could be the mechanism, by which the gene expression of these receptor genes is permanently altered due to the chronic stress exposure during adolescence? We previously reported lasting differences in glucocorticoid and mineralocorticoid receptor expression levels 12 months after stress exposure (Sterlemann et al., 2007). As both receptors are highly expressed in the hippocampus and act as transcription factors, they could lastingly influence the expression of target genes, including systems involved in memory function. Interestingly, both MR and GR are known to be involved in information processing and modulate hippocampal circuits that underlie complex cognitive function such as spatial learning. Indeed, their role in learning and memory could be well established by conducting studies using knockout lines or the administration of selective agonists or antagonists (Oitzl and de Kloet, 1992; Sandi and Rose, 1994; Tronche et al., 1999; Oitzl et al., 2001). Depending on the differential activation of MR and GR by corticosterone, LTP can be blocked or induced, suggesting MR and GR to be important determinants of synaptic plasticity and finally learning and memory (Joels and Krugers, 2007; de Kloet et al., 2002). In addition to the genomic actions of these receptors, the MR has recently also been implicated in fast, non-genomic glucocorticoid actions (Karst et al., 2005), which might also influence memory acquisition or retrieval. An alternative or additional possibility for the lasting changes in gene expression observed 12 months after chronic stress exposure are epigenetic modifications of the DNA. These modifications have recently been shown to be evoked and varied by environmental influences and result in enhanced or diminished transcription rates of specific genes (Agrawal, 2001; Weaver et al., 2004).

As mentioned above, structural changes of the hippocampal neurons could underlie the observed differences in memory function. Dendritic atrophy in terms of less branch points

and/or reduced dendritic length (Brunson et al., 2005; Conrad et al., 1996; Magarinos et al., 1996; McKittrick et al., 2000) as well as impaired neurogenesis have been observed following chronic stress in different species and across numerous paradigms (Joels et al., 2007; Mirescu and Gould, 2006). Although the structural analysis of the hippocampus in aged mice subjected to chronic stress during adolescence is beyond the scope of this study, our observation of pronounced differences in BDNF expression suggest the possibility of structural parameters underlying the functional deficits in previously stressed animals. Belonging to the family of the neurotrophines, which are strongly involved in neuronal survival and differentiation, BDNF is known to improve memory and its down-regulation in the hippocampus may be one major component causing LTP impairment. Studies using BDNF knockout mice proved BDNF being responsible for the induction and probably also maintenance of LTP (Figurov et al., 1996; Korte et al., 1995; Korte et al., 1996) that can be rescued by the application of exogenous BDNF in vitro (Patterson et al., 1996) and in vivo (Radecki et al., 2005). Aleisa and colleagues also observed reduced hippocampal BDNF levels and impaired LTP in the CA1 region in adult rats subjected to chronic psychosocial stress, underlining the strong detrimental impact of this type of stressor, leading to changes in neurotrophine levels and finally impaired cognitive function (Aleisa et al., 2006).

In summary, our results clearly demonstrate that chronic social stress exposure during the adolescent period of mice can result in long-term alterations of spatial memory performance, which are detectable even one year after the stress regimen was terminated. These behavioral differences are accompanied by differences in electrophysiological properties as well as a differential gene expression of genes involved in hippocampal memory function. These data underline the importance of the adolescent and young adult period as a critical transitory phase with potential for both adaptive and maladaptive outcomes in adulthood and indicate pathophysiological mechanisms that may contribute to the adverse long-term effects of chronic social stress on memory function.

4. Long-term consequences of chronic stress during adolescence on body fat distribution

In modern societies obesity is a seriously increasing public health issue. When defined by a body mass index of 30.0 or higher according to the National Health and Nutrition Examination Survey (NHANES9, its prevalence has increased by a twofold over the last 20 years, affecting about 30% of the US population (Tamashiro et al., 2007; Hu et al., 2004) with no trend for a decline (Hedley et al., 2004). The incidence of obesity is strongly associated with arterial hypertension and metabolic abnormalities as glucose intolerance, insulin resistance, increased visceral fat and dyslipidemia that, when clustering together, are referred to as metabolic syndrome (Day, 2007). These follow-on diseases associated with obesity impose a significant economic burden on individuals, families and public health systems (Ogden et al., 2007).

Chronic stress has been shown to represent a risk factor for obesity and the metabolic syndrome (Seematter et al., 2004) that adds to increased energy consumption and decreased physical activity in modern society. For instance chronic work stress was found to correlate with obesity in humans (Brunner et al., 1997), doubling the risk for metabolic syndrome (Chandola et al., 2006). A causal relation between hypothalamic-pituitary-adrenal (HPA) axis activity, obesity and specifically accumulation of visceral fat has been proposed (Rosmond and Bjorntorp, 1998). In a recent prospective study, Branth and colleagues demonstrated that in originally non-obese young men long-term stress induced abdominal obesity and symptoms of metabolic syndrome with a delay of about five months (Branth et al., 2007). Beside this epidemiological evidence for a linkage between stress exposure and metabolic changes in humans, various studies in animal models suggest that stress affects feeding behavior (Carr, 2002), alters caloric intake (Dallman et al., 2003) and changes body weight and fat content (Tamashiro et al., 2007).

A number of links exist between disturbed metabolic homeostasis and affective disorders. Next to the metabolic syndrome, chronic stress also represents a major risk factor for the development of affective disorders (de Kloet et al., 2005) and depressive patients more often suffer from general obesity, metabolic syndrome (Faith et al., 2002; Stunkard et al., 2003; Heiskanen et al., 2006) and cardiovascular complications (Perlmutter et al., 2000). Further HPA axis dysregulation can frequently be observed in major depression (Holsboer, 2000), and particularly patients with hypercortisolemia develop visceral obesity (Weber-Hamann et al., 2002). Therefore, a common pathophysiological basis of both affective disorders and the metabolic syndrome has been postulated (Kyrou and Tsigos, 2007).

Additionally, long-term effects of previous depressive episodes and psychosocial stress on the risk to develop metabolic syndrome or obesity have been concluded from clinical observations (Heiskanen et al., 2006; Raikkonen et al., 2007).
In the light of limited data explaining the pathophysiology of the delayed or prolonged impact of chronic stress on metabolism and body composition, preclinical studies using animals models for chronic stress in combination with non-invasive magnetic resonance imaging (MRI) are of particular use and can help to further elucidate the underlying mechanisms. The first goal of this study was to probe if chronic social stress applied during the adolescent period of mice has an effect on body fat distribution at an older age, i.e. 12 months after cessation of the stress regimen. Given the current opinion that restoration of the HPA axis function represents a common pathway of antidepressant drug action (Ising et al., 2007) and normalization of the HPA system has been shown to be a prerequisite for stable remission (Zobel et al., 1999; Holsboer, 2000), we further hypothesized that lasting effects of chronic stress on metabolic parameters may be prevented by a simultaneous chronic treatment with an antidepressant throughout the stress period.

Experimental design

Three different groups were used: (1) control animals, (2) untreated chronic stress animals and (3) chronic stress animals treated with the SSRI paroxetine (see drugs). Three weeks after the start of the chronic stress procedure, 12 animals of the chronic stress group were treated with the antidepressant paroxetine, while 20 animals remained untreated. At the start of the drug treatment animals of the chronic stress group showed clear symptoms of stress. Drug treatment continued for 4 weeks until the end of the stress exposure. Control animals (N = 22) were not treated with paroxetine. At the end of the stress procedure animals from all groups were separated and single housed. After 12 months of single housing with no further intervention, all animals were sacrificed under basal conditions.

Results

At the time of testing the three groups studied (controls, chronic stress and chronic stress + paroxetine) did not differ in mean body weight, or for visceral fat or subcutaneous fat content only. When analyzing the visceral-to-subcutaneous fat ratio we found significant group differences ($F(2,48) = 4.073$ ($p<0.05$)) (figure 20B). Post-hoc comparisons located the effect to the untreated stressed animals (1.571 ± 0.56) that differed significantly from

controls (1.21 ± 0.314; p<0.05) and paroxetine treated mice (1.23 ± 0.366; p<0.05). No difference was found between the control group and the paroxetine treated chronic stress animals (p = 0.93).

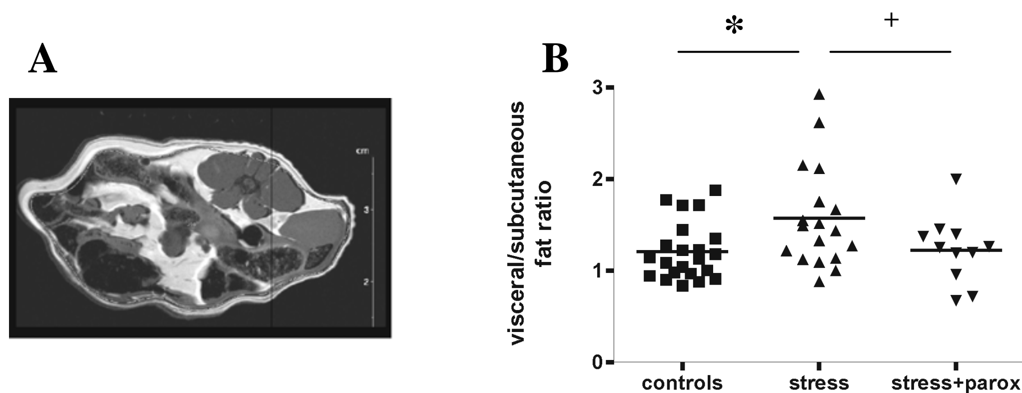

Figure 20: (A) Axial slice through the mouse abdomen. Fatty tissue is bright. A region-of-interest is indicated segmenting the image into visceral and (sub-)cutaneous compartments. (B) Visceral to subcutaneous fat ratio. Mean values for each group are indicated. * significant from control group. + significant from paroxetine treated chronic stress group.

Discussion

In this study we demonstrate for the first time long-lasting effects of early life chronic stress experience on body fat composition later in life in an animal model. At the age of 15 months, previously stressed animals showed a marked increase in the visceral to subcutaneous fat ratio. Intriguingly, these effects are very long lasting and observed months after the termination of the stressor under stress-free conditions. Our current results are further corroborated by findings of lasting effects of chronic stress in terms of long-term behavioral, neuroendocrine and neurogenetic alterations (Sterlemann et al., 2007). After one year of recovery baseline corticosterone secretion was not altered anymore, but we could still observe a decreased expression of hippocampal mineralocorticoid receptors. Thus, the chronic stress exposure during adolescence has permanently altered the expression of relevant genes in specific brain regions.

Which mechanism could account for the long-term effects of chronic social stress on body fat composition? It is easily conceivable that in addition to central effects chronic stress exposure has also affected peripheral glucocorticoid signalling, since we had observed a long lasting elevation of morning corticosterone levels and a flattened circadian rhythm in the animals of the chronic stress group (Schmidt et al., 2007). Especially a persistently altered expression of peripheral glucocorticoid receptors as a result of the long-term altered corticosterone levels is likely, which in turn might cause changes in downstream protein

expression or altered enzymatic activities. Mazusaki and colleagues could show that a transgenic mouse line which locally over expresses 11β hydroxysteroid dehydrogenase type 1 (11β HSD-1), an enzyme which catalyzes the regeneration of active glucocorticoids from inactive keto-forms, in adipose tissue displayed increased adipose levels of corticosterone and developed visceral obesity. As those mice also exhibited pronounced insulin-resistant diabetes and hyperlipidemia among other symptoms, the authors proposed that increased adipocyte 11β HSD-1 activity may be a common molecular etiology for visceral obesity and the metabolic syndrome (Masuzaki et al., 2001). This adipose tissue specific increase in 11β-HSD1 activity has recently be shown to be a common symptom in obese humans and rodents (Seckl and Walker, 2004). Thus, an increase in the activity of 11β HSD-1 may possibly also result from chronic stress exposure, which in our paradigm particularly is experienced in the highly adaptive and vulnerable period of adolescence and young adulthood. In addition, there are most likely numerous other changes in gene or protein expression of central or peripheral factors by chronic stress, determining the visceral fat content either directly or by influencing central body fat regulation, e.g. by the hypothalamus. As the animals of the chronic stress group who had received the antidepressant paroxetine (SSRI) during the stress phase did not show the increase in the visceral to subcutaneous fat ratio, one could rather propose central mechanisms being responsible for the changes in metabolic homeostasis. This hypothesis is supported by our earlier findings, where chronic treatment with paroxetine normalized several other centrally regulated HPA and behavioral parameters, like increased morning corticosterone levels, decreased hippocampal MR and GR expression and an increase in anxiety related behavior. Thus, there might exist converging underlying mechanisms that are responsible for both the development of depression and metabolic syndrome.

These experimental results have strong implications for clinical medicine at the crossing of neuroendocrinology, inner medicine and psychiatry. First, the findings help to bridge the gap between obesity and the metabolic syndrome which tend to appear in adulthood, and earlier exposure to chronic stress. Early life stress has for long been investigated mainly as risk factor for later development of affective disorders and other psychiatric conditions (de Kloet et al., 2005), but not for sustained or delayed metabolic changes that lack a behavioral correlate in the first place. Only in the last years, epidemiological evidence has emerged showing that depressive symptoms or very stressful life events increase the risk to develop type II diabetes and metabolic syndrome later in life (Raikkonen et al., 2007; Engum, 2007) and that major depression is predictive of long-term body weight variability

independent of medication effects (Hasler et al., 2005). Moreover, rather than clinical depression as such, hypercortisolemic states have been shown to aggravate the risk to disbalance metabolic homeostasis (Vogelzangs et al., 2007; Duclos et al., 2005) when analyzed cross-sectionally, suggesting a pathophysiological link. To the best of my knowledge, no clinical data have linked adolescent HPA axis profiles to metabolic data later in life. With regard to a translation to clinical medicine, our results should foster such longitudinal cohort studies. It should be noted that obesity itself is being recognized a chronic inflammatory state that through signalling of adipocytes may trigger further metabolic complications (Kyrou and Tsigos, 2007). Therefore, mechanisms such as epigenetic changes that adhere a metabolic risk situation once the stressor has ceased are of particular interest.

A second clinical implication arises from the results of the pharmacological intervention. The reconstitution of normal HPA axis signalling has been identified as a biological marker of successful antidepressant pharmacotherapy that also implicates a better clinical prognosis for a patient (Ising et al., 2007). Although premature at this stage, it may be speculated that early pharmacological targeting of the stress hormone system in affective disorders may also prevent later metabolic complications. Future work also needs to clarify if therapeutic consequences should be extended from psychiatrically manifest disease to situations of endocrinologically measurable chronic stress. With regard to their clinical implications, the data of this study are limited as visceral obesity was used as surrogate for metabolic disturbance while other parameters were not available.

Thus, stressful experiences during crucial life phases such as adolescence and young adulthood seem to highly affect body composition and therefore the risk for disease later in life. These data further support and confirm the sparse human data of chronic stress being a risk factor for altered body fat composition (Branth et al., 2007).

5. Rimonabant treatment during chronic stress: behavioral, neuroendocrine and metabolic consequences

The CB1 receptor is densely expressed in several brain regions including areas controlling emotional processes like the limbic system (Tsou et al., 1998) and there is substantial evidence proving the endocannabinoid system to be highly involved in the control of emotional behavior and mood (for review: Pacher et al., 2006). However, there are many contradictory findings on the specific way of action. On the one hand endogenous cannabinoids and the administration of exogenous cannabinoids like Δ9-THC were found to reduce anxiety-like behavior (Kathuria et al., 2003; Berrendero and Maldonado, 2002; Valjent et al., 2002) and on the other hand to enhance anxiety-related behavior in relevant test paradigms (Arevalo et al., 2001; Onaivi et al., 1990). These differences might in part derive from a dose dependency, but also test conditions and strain differences seem to have high impact on the behavioral phenotype. Also for selective CB1 antagonists, like for example rimonabant (SR141716A), the picture remains unclear. While in many studies an anxiogenic phenotype was described (Navarro et al., 1997; Arevalo et al., 2001), in some rodent anxiety and depression models the administration of rimonabant lead to anxiolytic and antidepressant-like effects (Rodgers et al., 2003; Akinshola et al., 1999; Haller et al., 2002). These inconsistent findings were more recently suggested to be due to the fact that cannabinoids exert their effects in a bidirectional manner: Via the CB1 receptor, resulting in an anxiolytic effect, and via a novel neuronal cannabinoid receptor, resulting in an anxiogenic effect (Haller et al., 2002; Rodgers et al., 2005). Accordingly, CB1 receptor knockout mice display generally marked increases in anxiety-related behavior compared to wild types (Haller et al., 2002; Maccarrone et al., 2002). Thus, as already stated by Rodgers and colleagues, to better characterize the effects of CB1 receptor blockade on emotional processes more ethologic-orientated techniques need to be employed (Rodgers et al., 2003). There are a series of studies that proved the endocannabinoid system to be involved in the modulation of the HPA axis, which is a major regulator of the stress response and has a crucial role in mood regulation. Thus, the potential functional relationship between the effects of endocannabinoids on the HPA axis and anxiety is under extensive research (Manzanares et al., 2004; Murphy et al., 1998; Patel et al., 2004; Barna et al., 2004). In a large-scale study, for instance, Griebel and colleagues investigated the effects of acute or chronic rimonabant treatment in several animal models of anxiety in rats, mice and gerbils. As one major finding in mice they could demonstrate a clear improvement of stress symptoms after chronic rimonabant treatment during a chronic unpredictable stress regimen

in terms of a reduced anxiety- and depression-like phenotype (Griebel et al., 2005). However, they did not investigate potential lasting effects of rimonabant on HPA function and behavior.

The CB1 receptor and the endocannabinoids are also known to be present in brain regions controlling food intake and are proposed to be involved in the modulation of feeding behavior (Cota et al., 2003; Mechoulam and Fride, 2001; Howlett, 2002). By performing CB1 antagonists studies, central orexigenic effects of the endocannabinoids could be revealed (Colombo et al., 1998; Freedland et al., 2000). Accordingly CB1 knockout mice are usually lighter and leaner compared to their wild type littermates (Cota et al., 2003). However, so far only little is known about the interaction of the endocannabinoid system with the numerous other neuropeptides involved in the regulation of feeding and energy balance. Gradually first evidence for a functional relationship between the endocannabinoid system and central metabolic regulators can be manifested: Leptin, which is suggested to be the key mediator of the anorexogenic drive, has been shown to lower central hypothalamic endocannabinoid levels (Di Marzo et al., 2001), while ghrelin, which is orexigenic, potentially acts by increasing endocannabinoid production in the hypothalamus (Tucci et al., 2004). To gain a comprehensive picture, though, further research is indispensable. Of late, the CB1 antagonist rimonabant is used successfully as an antiobesity drug and most likely exerts its function by affecting the HPA axis (Manzanares et al., 1999). Interestingly, both depression and obesity share a dysregulation in HPA axis activity and several antiobesity as well as antidepressant drugs affect HPA axis function (Bornstein et al., 2006). Thus, to better understand the role of the endocannabinoids in modulating the HPA axis would be of great value.

To further elucidate this interaction, in this study we investigated the impact of chronic CB1 antagonist treatment on our recently validated chronic social stress paradigm in mice. The stress is applied during the adolescent period of the animals, a phase where the brain is in a state of high plasticity, but also high vulnerability (McCauley et al., 1997). In the first part of this study we investigate the effect of chronic treatment with the CB1 receptor antagonist rimonabant (SR141716A) on the medium-term consequences of chronic stress (i.e. 7-15 days after stress cessation) on physiological, neuroendocrine and behavioral parameters. In the second part of this study, we investigated the efficacy of chronic rimonabant treatment during the stress period in preventing potential long-term metabolic alterations resulting from chronic stress. Therefore we subjected control and previously stressed animals at the age of 6 and 12 months, i.e. 3 and 9 months after cessation of the

stressor, to an oral glucose tolerance test and glucose as well as insulin levels were determined.

Experimental design

108 mice were chronically stressed, while 40 control animals remained undisturbed with the same cage mates throughout the experiment. 3 weeks after the start of the chronic stress procedure, 52 animals of the chronic stress group were treated with the CB1 antagonist rimonabant (SR 141716A) for 28 days. At the start of the drug treatment animals of the chronic stress group showed clear symptoms of stress. After 7 weeks of continuous stress exposure all animals were separated and since then kept in single housing with no further drug treatment.

Experiment 1: medium-term effects

Tests were carried out 7-15 days after the last change in group composition. A subset of 16 animals per group was used for neuroendocrine testing after 7 days of single housing. The fur state of the animals was rated by two independent researchers according to a 4-point scale (Mineur et al., 2003; Ducottet et al., 2003), with 1 as the best and 4 as the worst fur condition. On the next morning, the animals were decapitated under basal conditions.
The remaining animals were tested in the following behavioral tests: open field (after 8-9 days of single housing), novelty-induced suppression of feeding (after 10-11 days of single housing) and elevated plus maze (after 14-15 days of single housing).

Experiment 2: long-term effects

For the investigation of the long-term effects of chronic stress, a total of 30 (10 control, 10 chronic stress and 10 chronic stress/rimonabant) 6- or 12-month-old animals were used for oral glucose tolerance testing. All animals were food deprived overnight from 17.00h till 8.00h the next morning. At 8.00h one basal blood sample was taken by tail cut (Fluttert et al., 2000), followed by application via oral gavage of 3 mg/g bodyweight glucose dissolved in 250 µl water. Further blood samples were taken 30, 90 and 120 minutes after glucose administration again by tailcut. Glucose was determined immediately based on electronical sensor technology using a blood glucose meter (Ascensia Elite®, Bayer). For insulin blood samples were centrifuged and plasma was kept frozen until determination using a Lincoplex assay (LINCOplexTM, Linco research, Inc).

Results

Experiment 1

Physiological data

Body weight was determined at the end of the chronic social stress and treatment. One-way ANOVA revealed a significant difference in body weight between the groups ($F(2,144) = 3.443$ ($p<0.05$)) with the untreated stress animals being significantly heavier compared to rimonabant treated chronic stress animals. The mean body weight of control mice was in between both stress groups (table 4). The adrenal and thymus glands of 16 control, 16 untreated chronic stress and 16 rimonabant treated chronic stress animals, which were sacrificed after seven days of recovery, were dissected and weighed. For both organs, ANOVA revealed a main effect of condition (adrenals: ($F(2,39) = 10.982$ ($p<0.001$)); thymus: ($F(2,34) = 15.352$ ($p<0.001$)) (table 4). Chronic stress resulted in a significant increase in adrenal weights. This effect was even worsened by rimonabant treatment during stress exposure ($p<0.1$). Thymus weight was significantly reduced in the untreated chronic stress group compared to control animals. Rimonabant treatment had neither ameliorating nor deteriorating effect.

The fur condition of the animals was rated carefully by two independent observers according to a 4 point scale prior to blood sampling, with a higher number corresponding to a worse fur state. A significant deterioration of the fur state of both untreated and rimonabant treated chronically stressed animals compared to controls could be detected ($F(2,143) = 23.498$ ($p<0.001$)) (table 4).

Table 4: Influence of rimonabant treatment during chronic social stress exposure on body weight, adrenal weight, thymus weight and fur state. * significant from control group, $p < 0,05$; $^{+}$ significant from chronic stress group, $p<0.05$;

	control	chronic stress	chronic stress + rimonabant	F-value	p-value
body weight (g)	37,667 ± 0,46	38,546 ± 0,36	37,098 ± 0,43	3,443	<0,05
adrenal weight (mg/g bw)	0.111 ± 0.009	0.141 ± 0.006*	0.167 ± 0.007*$^{+}$	12,631	0,001
thymus weight (mg/g bw)	1.268 ± 0.067	0.841 ± 0.034*	0.871 ± 0.072*	12,929	0,001
fur state	1.28 ± 0.08	2.15 ± 0.11*	2,28 ± 0.1*	—	0,001

Neuroendocrine data

Basal corticosterone plasma levels were measured after seven days of single housing. One-way ANOVA revealed a statistical trend for corticosterone differences between the groups ($F(2,44) = 2.869$, ($p<0.1$)), with rimonabant treated stress animals having increased morning corticosterone levels compared to controls ($p<0.1$) (figure 21). Due to large standard deviations, no significant differences could be observed between the untreated chronic stress animals and the controls.

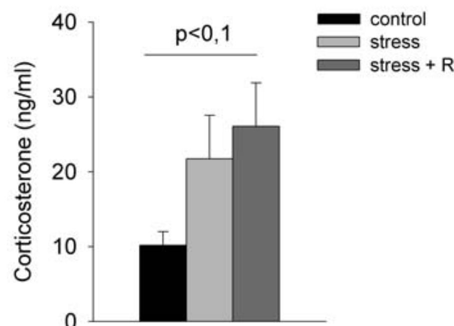

Figure 21: Influence of rimonabant treatment during chronic stress exposure during adolescence on HPA axis function: Basal morning corticosterone levels after one week of recovery. N = 16 per group.

Further, we analyzed basal gene expression of GR and MR mRNA in the hippocampus. For the GR, ANOVA revealed a significant effect of condition in the CA1 region ($F(2,29) = 5.214$ ($p<0.05$)) and the dentate gyrus ($F(2,29) = 5.539$ ($p<0.05$) (figure 22A). Chronic stress significantly decreased GR mRNA expression in the these regions, which could be partly prevented by treatment with the CB1 antagonist rimonabant. In the PVN no differences in GR mRNA expression levels could be found. For hippocampal MR mRNA, ANOVA revealed a significant effect of condition (CA1: $F(2,26) = 4.897$ ($p<0.05$); CA2: $F(2,26) = 5.338$ ($p<0.05$); CA3: $F(2,26) = 11.591$ ($p<0.001$); DG: $F(2,25) = 3.200$ ($p<0.1$)) (figure 22B). The mRNA expression level of MR in chronic stress animals was significantly decreased in the CA3 area compared to control animals, a statistical trend could be observed in CA2 ($p<0.1$). Rimonabant treatment did not reverse this effect, but on the contrary caused a stronger down-regulation, resulting in a significant decrease compared to the control group in the CA1, CA2 and CA3 region. A statistical trend could be detected in the dentate gyrus ($p<0.1$). We also analyzed basal gene expression of CRH in the whole PVN and AVP mRNA in the parvocellular part of the PVN, but could not detect any differences between the groups after seven days of single housing.

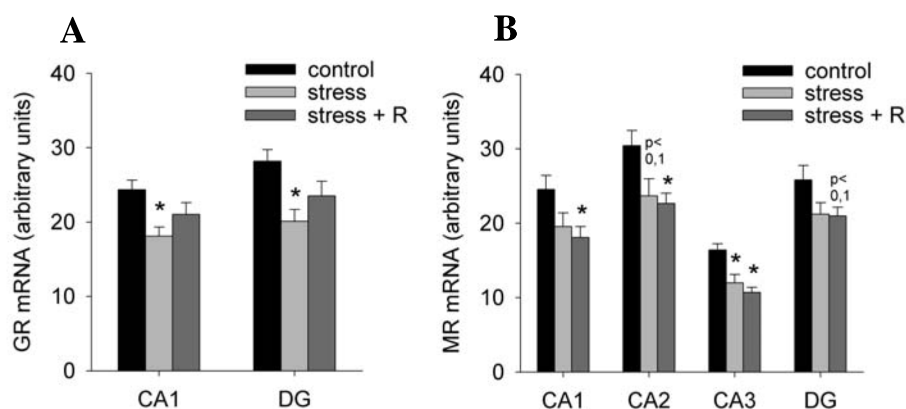

Figure 22: Influence of rimonabant treatment during chronic stress exposure on (A) GR mRNA expression in the CA1 region and the dentate gyrus of the hippocampus and (B) MR mRNA expression in the CA1, CA2, CA3 region and the dentate gyrus of the hippocampus. N = 12 per group. * significant from control group, $p<0.05$

Behavioral data

To asses medium-term alterations in behavior due to chronic stress exposure we tested the animals in anxiety-related behavioral tests. We recorded the locomotor activity of the different groups in an open field test. As a measure of the animals' adaptability to a novel environment, the distance traveled in the fifth minute of the test was subtracted from the distance traveled in the first minute, called locomotor adaptation. For this parameter, ANOVA revealed a main effect of condition ($F(2,35) = 8.399$ ($p<0.005$)) (figure 23A). Chronic stress resulted in a significant decrease of locomotor adaptation compared to controls ($p<0.01$). This effect could not be reversed by rimonabant treatment ($p<0.005$). The basal locomotor activity in the last 25 minutes of the open field exposure was not different between the groups ($F(2,35) = 0.540$, ($p = 0.588$)).

Untreated chronically stressed animals also displayed an enhanced anxiety-related behavior in the novelty-induced suppression of feeding paradigm. Control animals initiated the feeding of a snack faster compared to previously stressed animals with the rimonabant treated chronic stress animals being in between. However, due to large variation in the stress groups, this effect was not significant (ANOVA: ($F(2,34) = 1.709$ ($p = 0.197$)) (figure 23B).

In the elevated plus maze test a main effect of condition could be detected for the number of open arm entries ($F(2,30) = 5.517$ ($p<0.05$)) with untreated and rimonabant treated chronic stress animals exploring the open arms significantly less often compared to controls ($p<0.05$). Both groups also tended to spent less time on the open arm ($F(2,30) = 3.041$ ($p<0.1$)) (figure 23C). The total distance traveled did not differ between the groups.

Further, control animals showed significantly more head dips compared to chronic stress animals (ANOVA: (F(2,30) = 9.737 (p<0.001)); post hoc p<0.005) (figure 23D), which is regarded one of the best parameters of anxiety in the elevated plus maze (Fernandez, 1997). Also this effect was not reversed by rimonabant treatment.

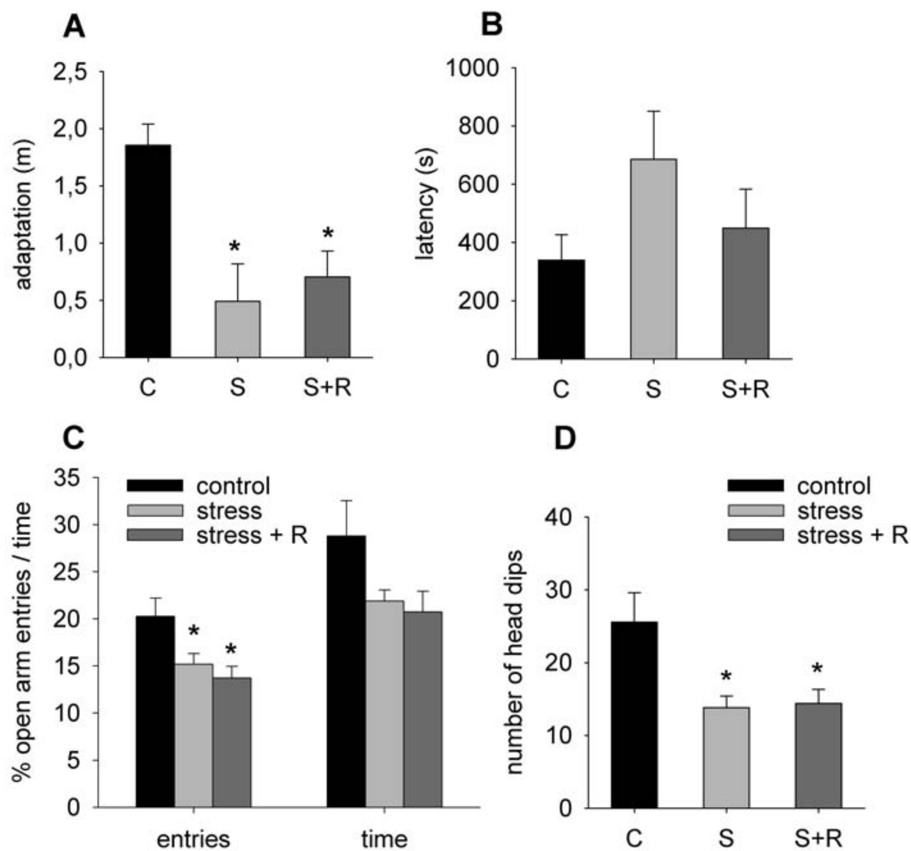

Figure 23: Influence of rimonabant treatment during chronic social stress exposure on changes in anxiety-related behavior. (A) Open field: Treatment effects on locomotor adaptation during the first 5 minutes. (B) Novelty-induced suppression of feeding: Treatment effects on the time till initiation of almond consumption. Plus Maze: Treatment effects on (C) time spent in the open arms and number of entries to the open arms and (D) on the number of head dips. control = C; chronic stress = S; chronic stress + rimonabant = S+R. N=12 per group. * significant from control group, p<0.05

Experiment 2

At the age of six month, i.e. after three months of recovery, glucose levels did not differ between the three groups at any time point of the oral glucose tolerance test (F (2,30) = 1.19 (p = 0.32)) (figure 24A). For corresponding insulin levels ANOVA with repeated measures revealed a main effect of time (F(2,23) = 35.06 (p<0.001)) and condition (F (2,23) = 17.452 (p<0.001)) (figure 24B). Via post-hoc analysis we determined significantly lower insulin levels in previously rimonabant treated chronic stress animals compared to

controls and untreated chronic stress animals 30, 90 and 120 minutes after glucose administration (p<0.05).

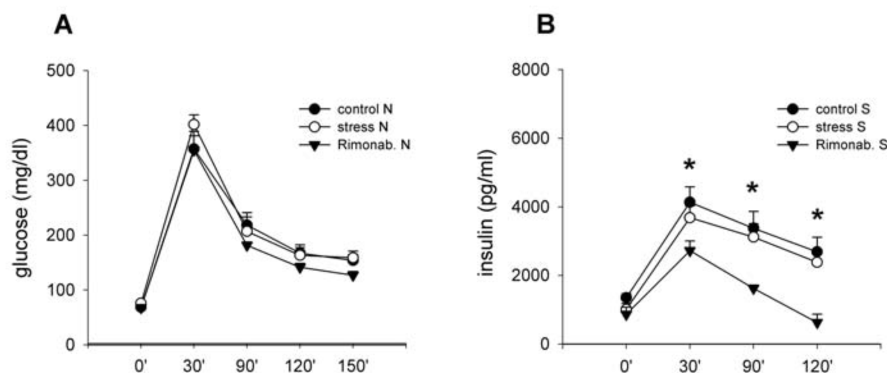

Figure 24: Influence of rimonabant treatment during chronic social stress exposure on long-term changes in glucose metabolism at the age of six months. Time course of (A) glucose levels during an oral glucose tolerance test and (B) corresponding insulin levels. N = 10 per group. * significant from control group, p<0.05

Also at the age of 12 months, i.e. 9 months after cessation of the stressor, no differences in glucose levels could be observed between the three groups ($F(2,29) = 1.58$ ($p = 0.225$)) (figure 25A). For insulin we detected a main effect of time ($F(2,26) = 26.95$ ($p<0.001$)) and condition ($F(2,26) = 9.259$ ($p<0.001$)) with the rimonabant treated stress animals having significantly lower insulin levels at all time points. Post hoc analysis revealed that rimonabant treated animals had significantly lower basal insulin levels compared to the untreated stress animals after 15 hours of food deprivation (p<0.05) and significantly lower insulin levels compared to controls (p<0.05) 30 and 90 minutes after the glucose administration (figure 25B).

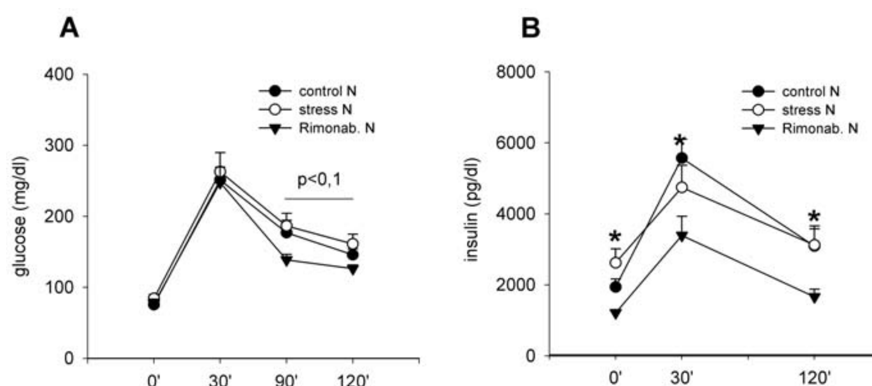

Figure 25: Influence of rimonabant treatment during chronic social stress exposure on long-term changes in glucose metabolism at the age of 12 months. Time course of (A) glucose levels during an oral glucose tolerance test and (B) corresponding insulin levels. N = 10 per group. * significant from control group, p<0.05

Discussion

In the present study we investigated whether the medium-term effects of chronic social stress obtained after at least one week of recovery can be prevented by treatment with the potential anxiolytic rimonabant during stress exposure. We chose the CB1 antagonist as it is well known that the endogenous cannabinoid system is highly involved in the modulation of the HPA axis (Murphy et al., 1998; Patel et al., 2004; Barna et al., 2004) and, although many studies have shown rather anxiogenic effects of CB1 antagonism (Navarro et al., 1997; Arevalo et al., 2001), there is also strong evidence for an anxiolytic capacity of this drug (Griebel et al., 2005; Rodgers et al., 2002). However, in our study the compound was not able to normalize the medium-term effects of chronic stress exposure on HPA axis function and behavior obtained 7-14 days after cessation of the stressor and treatment. Rimonabant did not prevent the animals from increased stress hormone levels, nor from adrenal enlargement and thymus shrinkage. Moreover, the adrenals of the rimonabant treated stress animals were even more enlarged compared to the ones of the untreated stress animals. This might be due to the fact, that under chronic treatment with the CB1 antagonist the dampening effect of the endogenous endocannabinoid system on the HPA axis is silenced for a long time and therefore the HPA activity may be even more increased in rimonabant treated animals compared to untreated stress animals. Additionally, the fur state of the CB1 antagonist treated animals was deteriorated to the same extent as the fur state of the untreated chronic stress animals, indicating a reduction in grooming behavior, which is associated with a depression-like phenotype (Mineur et al., 2003). Further, rimonabant treatment did not affect or avert the stress induced down-regulation of hippocampal MR, but it did partly prevent GR down-regulation in the CA1 region and the dentate gyrus of the hippocampus. This resembles a similar situation we have observed in our model after treatment with the selective CRHR1 antagonist DMP696 during stress exposure, where we could also detect a selective prevention of GR down-regulation, with no effect on MR expression levels (Schmidt et al., 2007). On the other hand, this is the converse effect we observed after treatment with the SSRI paroxetine, where we could observe even an increase in MR expression levels with no effect on GR down-regulation, suggesting elementary differences in the underlying central pathways involved in their mechanism of action (Schmidt et al., 2007). Nevertheless, the rimonabant induced prevention of GR down-regulation and the resulting presumably improved negative feedback could not counter regulate the largely increased corticosterone levels. In addition, rimonabant was not able to reverse the increased anxiety phenotype observed in chronic

stress animals 7-14 days after recovery in the elevated plus maze and in the open field test, with all three groups displaying similar general locomotor activity. Only in the novelty-induced suppression of feeding paradigm a slight improvement in terms of shorter feeding latencies could be detected. Due to a large standard deviation in all three groups, though, this effect was not significant. These findings are in sharp contrast to the data of Griebel and colleagues, who could, after chronic rimonabant treatment with the same dose (i.e. 10 mg/kg per day p.o.) during chronic mild stress, observe both improvement of the fur state and a reduction in the stress induced anxiety in the elevated plus maze (Griebel et al., 2005). However, in this study the animals were tested during the stress phase and under treatment, so the alterations in the anxiety-related behavior might be rather attributable to the direct effects of the drug than to an efficacious prevention of negative consequences originating from the chronic stress paradigm. Another important finding was described by Rodgers and colleagues, who reported reduced anxiety-related behavior in the plus maze in acutely rimonabant treated animals only in maze experienced and not in maze naïve mice and only when treated with a specific dose (i.e. 1 mg/kg i.p.) (Rodgers et al., 2003). As one potential interpretation of this pattern the authors suggested that 'trial 2' anxiety differs qualitatively from 'trial 1' anxiety, a hypothesis that is supported by the known 'one trial tolerance' of several anxiolytics such as diazepam (File and Zangrossi, Jr., 1993; Rodgers and Johnson, 1998). Therefore, the initial exposure may fail to recruit, while the different experience in 'trial 2' might activate the endocannabinoid system, what is an obvious prerequisite for the effectiveness of a selective antagonist. Further evidence for this hypothesis comes from Marsicano and colleagues who recently reported that re-exposure of mice to learned fear cues selectively increase endocannabinoid levels in the basolateral amygdala (Marsicano et al., 2002). Therefore it might be possible that in our study existing alterations following rimonabant treatment during stress could have been observed in re-test exposures, but not in single trials. Moreover, as rimonabant was recently found to be also able to reduce anxiety-related behavior in CB1 knockout mice (Haller et al., 2002), the existence of a second rimonabant sensitive receptor becomes more and more likely. It is assumed that CB1 and the potential novel receptor exert opposing effects on anxiety-related behavior, with activation of CB1 being anxiolytic and activation of the novel receptor being anxiogenic. Thus, in initial trials of anxiety-related tests both receptors might be blocked to the same extend thereby neutralizing their effects. Only after desensitization of CB1 regulated mechanisms in the initial trial, in a second trial predominantly the novel,

potentially anxiogenic receptor is blocked, resulting in an anxiolytic-like effect on the behavior (Rodgers et al., 2003).
In conclusion, besides some positive exceptions, like hippocampal GR mRNA expression, or negative exceptions, like adrenal enlargement, in our chronic social stress paradigm chronic treatment with rimonabant had neither long-term beneficial nor maladaptive effects on a physiological, neuroendocrine and behavioral level.
In the second part of this study we determined the potential long-term consequences of the chronic social stress regimen on the animals' glucose clearance in an oral glucose tolerance test and their respective insulin levels at the age of six and 12 months, as we had evidence for long-term metabolic changes at an older age resulting from chronic stress from previous studies. For instance, via magnet resonance imaging at the age of 15 months, i.e. after one year of recovery, we could detect alterations in the fat distribution of the animals with the previously stressed ones having an increased ratio of visceral to subcutaneous fat compared to controls. These changes could be prevented by chronic treatment with the SSRI paroxetine during the stress phase (see project 4). In humans, visceral adiposity is a well known risk factor for the development of metabolic syndrome, which is also highly associated with changes in insulin sensitivity and diabetes type II. However, in this study no differences in glucose nor in insulin levels could be detected between animals of the chronic stress and the control group at the age of six or 12 months. A preliminary result from pilot studies, where chronic stress animals seemed to have lower insulin levels after glucose administration in an equally performed glucose tolerance test, could therefore not be corroborated. Hence, alterations in body fat distribution seem therefore not to be associated with changes in other metabolic parameters like elevated glucose or insulin levels. Further, we investigated whether the chronic rimonabant treatment during the stress phase might have a beneficial long-term effect on the animals' metabolism and is able to prevent potential maladaptive consequences. The efficacy of this drug on normalizing body weight and metabolic parameters is well established in animals models of obesity: after chronic treatment a significant weight loss and reduction in food intake can be observed, accompanied by lowered plasma leptin, insulin and fatty acid levels (Ravinet et al., 2003; Poirier et al., 2005). The same picture holds true for CB1 knockout mice, which are lighter and leaner compared to their wild type controls, accompanied by reduced plasma insulin and leptin levels (Ravinet et al., 2004). Recently, also in humans rimonabant was introduced as an antiobesity drug (Acomplia®, Sanofi Aventis) leading to improvement of lipid and glucose metabolism and an improvement of food intake and energy metabolism

(Henness et al., 2006). In our study in both oral glucose tolerance tests, performed at the age of six and 12 months respectively, i.e. after three and 9 months of recovery, glucose levels did not differ between the groups throughout the test. However, corresponding insulin levels were indeed lower in rimonabant treated chronic stress animals compared to the control and the untreated chronic stress group at both the age of six months and 12 months. At both time points the animals of all groups did not differ anymore in their mean body weight. Despite their lower insulin levels, the animals of the rimonabant group catabolized the glucose at a similar rate compared to the other two groups, suggesting that chronic treatment with rimonabant during the adolescent and young adult period of the animals had persistently altered the insulin sensitivity of the animals. The mechanisms involved are certainly manifold, as the CB1 is ubiquitously expressed in organs involved in the regulation of energy homeostasis, such as adipose tissue, muscle, liver and gastrointestinal tract (Ameri, 1999). The altered insulin sensitivity might be in part attributable to increased adiponectin levels in adipose tissue resulting from CB1 blockade as it could be observed in obese fa/fa rats (Bensaid et al., 2003), but also enhanced glucose uptake in the muscles and reduced lipogenesis in the liver are likely to play a role (Woods, 2007). Thus, this is the first study demonstrating long-term beneficial effects of rimonabant on insulin sensitivity after a relatively short treatment time (four weeks). In a clinical study, Blüher and colleagues demonstrated that circulating endocannabinoids are positively correlated with visceral fat mass and fasting plasma insulin concentrations, whereas CB1 mRNA expression was negatively correlated with visceral fat mass (Blüher et al., 2006). A dysregulation of the peripheral endocannabinoid system seems therefore to be associated with abdominal fat accumulation and resulting metabolic changes that may finally lead to the development of metabolic syndrome. Thus, the endocannabinoid system represents a promising target for the treatment of obesity. However, as the CB1 is ubiquitously expressed in the CNS and in the periphery, it is currently not possible to target only the sites involved in energy homeostasis. Hence, a considerable number of side effects could be observed in patients taking rimonabant, among them increased anxiety and depression (Henness et al., 2006). Therefore it remains to be seen, if the advantages outweigh the potential disadvantages and the subsets of obese patients receiving the drug need to be carefully evaluated.

IV Discussion

7.1 Discussion
7.2 Conclusions
7.3 Future perspectives

7.1 Discussion

The aim of this thesis was the validation of a novel chronic social stress paradigm in male CD1 mice with regard to face, predictive and construct validity (see introduction for definition) and to further characterize the resulting long-term consequences on neuroendocrine, behavioral and cognitive function as well as alterations in metabolic parameters.

This paradigm shows a high degree of construct validity, mimicking the human situation of chronic social stress very closely. The social stress situation is unavoidable for the animals and constantly present at every time of the day. Importantly, instead of adapting to the situation, the stressfulness of living without clear hierarchical orders seems to increase over time. As this paradigm is performed during the adolescent and young adult period, it also covers a very important stage of life of the animals and is indeed long enough (seven weeks) for effectively resembling a chronic exposure. With our novel animal model, we could demonstrate a high degree of face validity for chronic stress, as we found clear and robust signs of chronic stress exposure (project 1 and 2). Animals subjected to the chronic stress exposure had larger adrenals and a smaller thymus gland, both physiological correlates of prolonged activation of the HPA axis. Accordingly, morning corticosterone levels where found to be largely increased at the end of the stress period. Interestingly, the amount of secreted ACTH was similar between both groups, indicating an enhanced adrenal sensitivity in chronic stress animals. We also observed a flattening of the circadian rhythm of the animals, a phenomenon which has also been described in depressed patients (Deuschle et al., 1997; Weber et al., 2000). The changes in peripheral parameters of the HPA axis are paralleled by differences of central gene expression in brain areas involved in stress regulation. Most prominently, the expression of mineralocorticoid receptors and glucocorticoid receptors in the hippocampus was found to be decreased following chronic stress exposure. This finding is in line with previous reports, which also indicated a decreased expression of these two receptor subtypes after social stress in tree shrews (Meyer et al., 2001). Further, in line with previous evidence we detected an increased expression of vasopressin in the paraventricular nucleus, which supports the notion that under chronic stress conditions vasopressin plays an increasingly important role in regulating HPA axis function (Aguilera, 1994; Volpi et al., 2004).

Chronic stress exposure also affects the behavior of the animals. Most animal models for chronic stress focus on the adverse consequences on the anxiety- and depression like phenotype, based on the vast evidence linking stress and affective disorders (Schmidt and

Müller, 2006). Predominantly unconditioned anxiety-related behavior seems generally increased as a consequence of exposure to chronic stress, thereby supporting epidemiological data of an increased risk of anxiety disorders in chronically stressed individuals. The data obtained with our model support this hypothesis, as exposure to chronic social stress resulted in increased anxiety-like behavior. For instance in the novelty-induced suppression of feeding test, where the animals are presented with a piece of palatable food (e.g. almond) in a novel environment, chronic stress animals spend significantly more time until initiating food consumption compared to unstressed controls. These data are confirmed by results from the elevated plus maze, where animals subjected to chronic stress spend less time on the open, unprotected arms compared to controls, indicating increased anxiety. In addition, stressed animals display a significantly deteriorated fur quality, indicating a decrease of self care and grooming, which has been rather associated with depression-like behavior (Ducottet and Belzung, 2005).

As pointed out previously, in humans chronic stress is generally not sufficient to induce depression, but it is merely one of many possible risk factors. An useful animal model for depression should not only include chronic stress as an environmental risk factor, but also other environmental or genetic risk factors, which would further enhance the vulnerability of the individual. It is thus very important to differentiate between animal models for chronic stress and animal models for a specific disease as major depression. While the first is readily possible and many good animal models for chronic stress exist, the latter seems problematic.

An important issue that needs to be addressed when considering face validity is the persistence of symptoms after chronic stress exposure. The majority of studies investigated only effects during or right after the end of the stressor, thereby focusing more on acute effects. However, in humans the maladaptive responses to chronic stress often outlast the actual stress phase by many years or even decades. Only few preclinical studies examined the long-term effects of acute or chronic stressors. Vallès and colleagues investigated the long-term consequences of a single immobilization stress on gene expression in several brain areas, demonstrating lasting differences in stress-induced gene expression (Valles et al., 2003; Valles et al., 2006). Analogously, a single exposure to social defeat stress has also been shown to have persistent electrophysiological consequences (Artola et al., 2006). Few studies have also indicated that some of the observed neuroendocrine and behavioral effects evoked by chronic stressors persist even if the stress is discontinued, which is a crucial factor with regard to human pathology (Tsankova et al., 2006). With our new animal

model for chronic social stress, we could observe clear effects of the stress exposure days, weeks or even months after the termination of the stressor. For instance, certain aspects of anxiety-related behavior have still been found altered even 12 months after stress exposure (project 2). However, these long-lasting effects are not generalized to all aspects of anxiety, but remain defined to specific behavioral domains. Interestingly, the individual variation of the persistent and long-term effects of chronic stress exposure is much higher compared to the acute and medium-term effects, indicating that some animals had recovered from the stressful experience, while others remained affected for a long time. Additionally, at an older age also alterations in the cognitive function of previously stressed animals (project 3) could be detected. These are manifested in an inferior performance in spatial memory tests compared to controls paralleled by reduced in vitro LTP. Also metabolic parameters like body fat distribution were found altered several months after cessation of the stressor (project 4) implying a long-lasting high impact of the stress regimen on the metabolism of the animals.

The third main validity criterion for animal models is the predictive validity. With our model, we could demonstrate that many of the persistent effects of chronic stress exposure can be prevented by simultaneous pharmacological treatment with different types of antidepressants: the serotonin reuptake inhibitor paroxetine (project 1 and 3) and the novel non-peptide CRH-receptor 1 antagonist DMP696 (project 1). Although both drugs were effective in preventing or ameliorating the stress effects, the mechanism of action underlying is probably very different. Especially for the CRHR1 antagonist it seems likely that the treatment effects are based on a specific suppression of the CRH-mediated effects of stress exposure in the brain and the periphery. On the contrary, treatment with the potentially anxiolytic CB1 antagonist rimonabant did not improve stress symptoms (project 5). However, more drugs and compounds from different classes of drugs need to be tested in this model before the claim of good predictive validity could be fully made. Further, as we observed clear long-lasting effects of the chronic stress exposure, a treatment starting after the termination of the stress paradigm would probably resemble the human situation more closely.

Taken together, the recently developed chronic social stress paradigm in mice provides a useful basis to further elucidate the neurobiological mechanisms underlying the postulated causal relationship between chronic stress and the development of human affective disorders. A better understanding of those pathophysiological mechanisms, in turn, will

contribute to the development of novel, innovative drugs in the future, especially in the field of antidepressants.

7.2 Conclusions

The following conclusions may be drawn from the results presented in this thesis:

(1) The chronic social stress paradigm is a valid animal model for investigating symptoms of stress-related disorders

(2) The chronic stress regimen evoked severe and long lasting changes on a physiological, neuroendocrine and behavioral level

(3) The chronic stress experience had influence on cognitive function in aged animals

(4) Chronic stress during adolescence is a risk factor for metabolic alterations later in life

(5) Rimonabant can not be utilized as an anxiolytic or antidepressant drug in this paradigm

7.3 Future perspectives

A main advantage of this novel animal model for chronic stress compared to other rodent models is the possibility to conduct large-scale studies, where a high number of animals can be stressed simultaneously. A rate limiting factor is now the throughput of tests or measurements (behavior, physiology etc.), rather than the availability of stressed and control mice. By using this model in large-scale approaches, it is thus now possible to study the issue of individual vulnerability to chronic stress by comparing the individual responses (acute and long-term) within the chronic stress group and to detect new potential target genes that are involved either in the process of recovery or in the maintenance of prolonged stress symptoms. It seems clear that in order to mimic the human situation of chronic stress exposure and the consequent increased risk for diseases in animals, it is necessary to incorporate the issue of stress vulnerability. A better understanding of why a certain combination of genetic and environmental risk factors together with individual life history leads to a specific disease is essential for the study of successful treatment strategies. Future research will therefore have to focus more on the individual differences in being resistant to stressful situations. The development of a novel mouse paradigm for chronic stress, which

offers the possibility to work with a high number of animals, is thus a first step in this direction and will open up new research possibilities.

Summary

Besides genetic risk factors, increasing evidence shows an important role of environmental factors as stressful life events for the development of metabolic, cardiovascular and affective disorders. Particularly chronic stress and the resulting characteristic dysregulation of the hypothalamic-pituitary-adrenocortical (HPA) axis are regarded as key risk factors for a variety of diseases, including major depression and anxiety disorders. As experimental variation of early environment and stress exposure cannot be performed in humans for ethical reasons, preclinical studies using animal models are indispensable to improve our understanding of the consequences of chronic stress.

A large number of paradigms have been established to induce chronic stress in rodents. However, many of these paradigms do not consider the etiology of human stress-associated disorders, where the stressors involved are mostly of a social nature and the effects of the stress exposure persist, even if the stressor is discontinued. In addition, many chronic stress paradigms are problematic with regard to stress adaptation, continuity, duration and applicability.

To overcome these limitations, this doctoral thesis develops and evaluates a novel chronic social stress paradigm in male CD1 mice, which is based on a chronically unstable social environment. As many psychiatric studies indicate that childhood and adolescence are periods of high stress and trauma vulnerability, the animals are exposed to the stressor during their adolescent and young adult period. This chronic social stress regimen lasts continuously for seven weeks and results in long-term physiological, neuroendocrine and behavioral changes. It fulfills the criteria of face, construct and predictive validity for animals models of chronic stress, thereby mimicking the human condition of stress-related disorders with respect to its etiology, symptomatology, treatment and behavioral basis. The model is further suitable to investigate the interaction between genetic susceptibility and environmental factors.

Part I introduces the topic by describing the function of the stress system and its components, with CRH being one of the key regulators of the stress response, orchestrating neuroendocrine, autonomic and immune processes in response to stress. After that, the chapter approaches the function of MR and GR in the negative feedback regulation of the HPA axis. Further, chronic stress is described as a risk factor for a variety of diseases, and several existing animals models for chronic stress are summarized. Also the key issue of individual vulnerability is discussed briefly. Finally, the objectives of the thesis are formulated, with a primary focus on the validation of the novel animal model. Furthermore,

the resulting long-term consequences on neuroendocrine, behavioral and cognitive function, as well as on metabolic parameters, are to be characterized.
In project 1 the physiological, neuroendocrine and behavioral alterations obtained 7-14 days after cessation of the stress regimen, i.e. after 7-14 days of recovery, are described. Animals subjected to the chronic stress exposure displayed severe signs of stress in terms of increased corticosterone levels, increased adrenal and decreased thymus weights and a down-regulation of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNA levels. Also anxiety-related behavior in the elevated plus maze, the open field and the novelty-induced suppression of feeding paradigm was affected. Most consequences evoked by the chronic social stress regimen could be prevented by simultaneous, chronic treatment, with either the selective serotonin reuptake inhibitor (SSRI) paroxetine or the corticotropin releasing hormone receptor 1 antagonist DMP696. Corticosterone levels and organ weights were comparable to control ones and also the phenotype observed in chronic stress animals in the open field and in the novelty-induced suppression of feeding test could be reversed. By determination of hippocampal MR and GR mRNA expression levels, the different underlying mechanisms of action became apparent: paroxetine treatment mainly affected MR expression, while DMP696 selectively prevented GR down-regulation.
In project 2 the investigation of the persistent effects of chronic stress is continued by extending the recovery phase to 12 months. Physiological, neuroendocrine and behavioral parameters were assessed one year after stress termination and summarized in comparison to the immediate effects obtained shortly after cessation of the stressor. Although the changes of many parameters were far more pronounced when assessed immediately at the end of the stress phase, even one year after termination of the stressor significant differences between the previously stressed animals and their respective controls could be detected. For instance, MR was still found down-regulated in the CA3 region and in the dentate gyrus of the hippocampus, suggesting a persistent change in gene expression profile. Additionally, animals of the chronic stress group still displayed a more anxious phenotype in the elevated plus maze and the open field test. However, individual variation of the persisting effects was much higher compared to the results observed immediately or 7-14 days after stress termination, indicating that some animals had recovered from the stress experience, while others remained affected throughout their life span.
In project 3 we have focused our attention on the impact of the chronic stress phase during adolescence on the cognitive function of the animals at an old age, as we had detected persistent alterations in MR expression, which is known to be involved in information

processing. One year after cessation of the stressor, animals of the chronic stress group performed significantly worse in the Y-maze and the Morris water maze tests, both investigating spatial memory. In the object recognition and social discrimination test, no differences could be detected. The deficits in the spatial memory were paralleled by reduced in vitro LTP, measured in the Schaffer collateral pathway of the hippocampal CA1 region. Furthermore, the mRNA expression levels of the neurotrophic factor BDNF were found to be decreased in the hippocampus and the cortex. Those pronounced differences in BDNF expression suggest that structural parameters underlying the functional deficits in previously stressed animals might contribute to the weaker LTP. Additionally, expression levels of several neurotransmitter receptor subunits, which are known to play a key role in hippocampal learning and memory, such as GABA A and AMPA 1, were altered, certainly also contributing to the impaired memory function.

Chronic stress is also known to be a risk factor for metabolic diseases. In project 4 we therefore assessed potential metabolic alterations resulting from the chronic stress experience in animals at an older age. Therefore the body fat distribution of subcutaneous and visceral fat was determined via functional magnet resonance imaging (FMRI) in 15-month- old mice. An increased ratio of visceral to subcutaneous fat could be detected in previously stressed animals with no differences in body weight compared to controls. Interestingly, also these metabolic alterations could be prevented by antidepressant treatment with paroxetine, applied during the stress phase.

To study the crucial link between affective disorders and metabolic diseases, in project 5 the connection between the cannabinoid system and the stress system was further examined. Therefore, the efficacy of chronic treatment with the selective cannabinoid receptor 1 (CB1) antagonist rimonabant during the chronic stress period to prevent physiological, neuroendocrine and behavioral alterations observed in untreated stress animals was assessed. 7-14 days after cessation of the stress regimen, i.e. after 7-14 days of recovery, thymus weight of rimonabant-treated animals was diminished and their adrenals were found to be even more enlarged compared to untreated stress animals and controls. Also corticosterone levels were highly increased, and hippocampal MR mRNA down-regulation could not be averted. Only GR mRNA down-regulation in the CA1 region and the dentate gyrus of the hippocampus was partly prevented. In addition, rimonabant was not able to reverse the increased anxiety phenotype observed in chronic stress in the elevated plus maze and in the open field test. In the novelty-induced suppression of feeding paradigm a slight improvement in terms of shorter feeding latencies could be detected.

Taken together, in our paradigm rimonabant treatment during the stress phase was not very efficacious in preventing or reversing stress symptoms on the above mentioned levels. To further characterize potential changes in metabolic parameters, the animals were subjected to an oral glucose tolerance test at an older age, and their glucose and corresponding insulin levels were measured. However, contrary to preliminary results from pilot studies, no differences in glucose nor in insulin levels could be detected between animals of the chronic stress and the control group at the age of six and 12 months. However, in the previously rimonabant treated animals, insulin levels were indeed lower, compared to the controls and the untreated chronic animals with no differences between the groups in corresponding glucose levels. This may indicate that chronic treatment with rimonabant during the adolescent and young adult period of the animals had persistently altered the insulin sensitivity of the animals.

Part IV summarizes and discusses the results of this thesis in its entirety. It is concluded that the chronic social stress paradigm is a valid animal model for investigating symptoms of stress-related disorders with regard to face, predictive and construct validity. The chronic stress regimen evoked severe and long lasting changes on a physiological, neuroendocrine and behavioral level and further affected cognitive function and metabolic parameters of the animals at an older age.

With respect to the postulated causal relationship between chronic stress and the development of various human diseases, including affective disorders, these data establish the basis to study the underlying neurobiological mechanisms in more detail. A better understanding of those pathophysiological mechanisms, in turn, will contribute to the development of novel, innovative antidepressant drugs in the future. In order to mimic the human situation of chronic stress exposure and the consequent increased risk for diseases in animals more closely, it is now possible and necessary to incorporate the issue of individual stress vulnerability by comparing the individual responses (acute and long-term) within the chronic stress group by using this model in large-scale approaches.

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List of abbreviations

ACTH	adrenocorticotropic hormone
AMPAR	ionotropic glutamate receptor with α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid as its synthetic agonist
ANOVA	analysis of variance
AP-1	activating protein 1
AVP	arginine vasopressin
BDNF	brain derived neurotrophic factor
BSA	bovine serum albumine
CA	cornu ammonis (part of the hippocampal formation)
CB1	cannabinoid receptor 1
CMS	chronic mild stress
CNS	central nervous system
CREB	cAMP response element binding protein
CRH	corticotropin releasing hormone
CRHR	corticotropin releasing hormone receptor
DG	dentate gyrus
FMRI	functional magnet resonance imaging
GABAR	class of receptors that respond to gamma amino butyric acid
GR	glucocorticoid receptor
GRE	glucocorticoid response element
HPA axis	hypothalamo pituitary adrenal axis
i.p.	intraperitoneal
ITI	intertrial interval
LTP	long-term potentiation
MR	mineralocorticoid receptor
NFκB	neurotrophic factor kappa B
NMDA	N-Methyl-D-Aspartat
NR	NMDA-type ionotropic glutamate receptor with N-Methyl-D-Aspartat as its specific agonist; NRs are heteromers consisting of the subunits NR1 and various subunits of NR2, with the subunits NR2A and NR2B being the most important subunits
p.o.	per oral
POMC	preopiomelanocortin

PVN	paraventricular nucleus of the hypothalamus
SEM	standard error of the mean
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
V1b	vasopressin receptor 1b
11β HSD-1	11β hydroxysteroid dehydrogenase type 1

Assertation / Erklärung

Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt.

Des Weiteren erkläre ich, dass ich nicht anderweitig ohne Erfolg versucht habe, eine Dissertation einzureichen oder mich der Doktorprüfung zu unterziehen. Die vorliegende Dissertation liegt weder ganz, noch in wesentlichen Teilen einer anderen Prüfungskommission vor.

Ort, Datum	Unterschrift Vera Sterlemann

List of publications

Schmidt MV, Levine S, Alam S, Harbich D, **Sterlemann V**, Ganea K, de Kloet ER, Holsboer F and Müller MB
Metabolic signals modulate hypothalamic-pituitary-adrenal axis activation during maternal separation of the neonatal mouse.
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Sterlemann V[a], Schmidt MV[a], Ganea K, Liebl C, Alam S, Harbich D, Greetfeld M, Uhr M, Holsboer F and Müller MB
a) both authors equally contributed to this work
Persistent neuroendocrine and behavioral effects of a novel, etiologically relevant mouse paradigm for chronic social stress during adolescence
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Sterlemann V, Ganea K, Liebl C, Harbich D, Alam S, Holsboer F, Müller MB and Schmidt MV
Long-term behavioral and neuroendocrine alterations following chronic social stress during adolescence: implications for stress-related disorders
Hormones and behavior, 2007; doi:10.1016/j.yhbeh.2007.11.001

Curriculum vitae

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