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Long-term effects of chronic unpredictable mild stress on cell adhesion molecules NCAM and L1 in the regulation of behaviour in C57BL/6J mice

Dissertation

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I INTRODUCTION

1.1 Cell adhesion molecules

Cell adhesion molecules play a pivotal role in the organisation of individual cells into functional tissues providing cell-cell and cell-matrix interactions. In the nervous system cell adhesion molecules are important for embryonic development, regulating neuronal proliferation, migration, survival, neurite outgrowth and axonal guidance. In the developing and adult brain, cell adhesion molecules initiate and organise synapse formation and synaptic plasticity (Maness and Schachner, 2007). Alterations in these processes lead to severe human developmental diseases as well as stress-related mood disorders (Sandi and Bisaz, 2007).

Cell adhesion molecules are divided into three major families: the integrins, the cadherins and the immunoglobulin (Ig) superfamily. Members of immunoglobulin superfamily contain at least one Ig like-domain which provides specific interaction with binding partners in a calcium independent manner. The immunoglobulin superfamily is further subdivided according to the presence of other functional domains like fibronectin type III repeats (originally identified as motifs in the extracellular matrix) molecule fibronectin, (Kornblihtt et al., 1985), catalytic domains, and type of attachment to the cell membrane (Brümmendorf and Rathjen, 1996; Fig.1). Immunoglobulin superfamily members often undergo extensive posttranslational modifications like phosphorylation, palmitoylation and glycosylation.

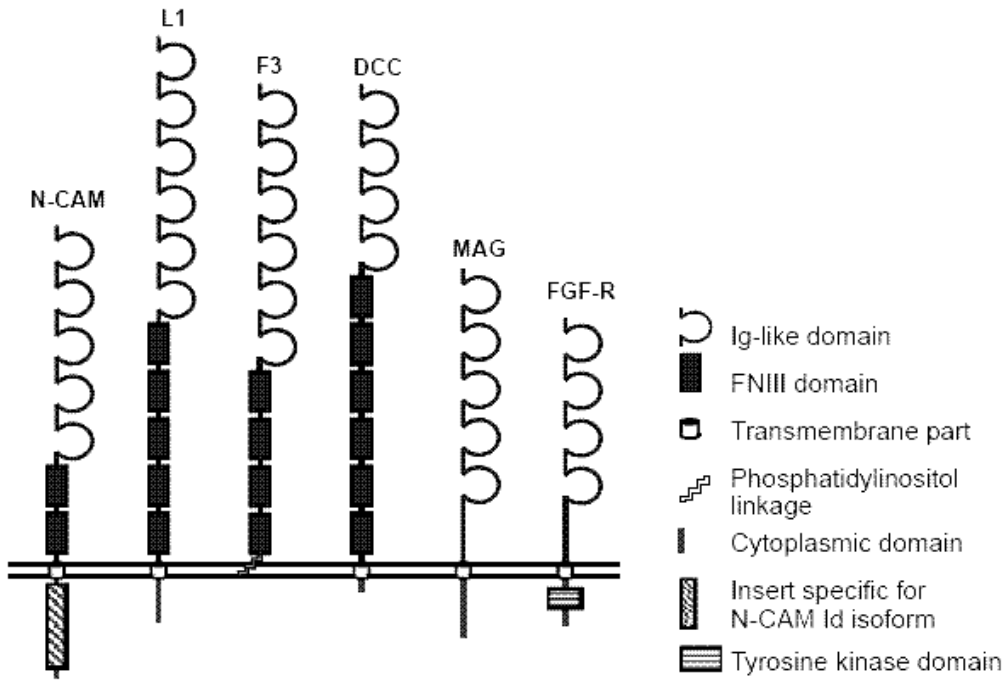

Figure 1. Representatives of different subgroups of the immunoglobulin (Ig) superfamily of cell adhesion molecules. Members of the Ig superfamily consist of an extracellular domain containing Ig- like domains. The rest of the molecule may include different domains like fibronectin type III (FNIII) repeats and, for transmembrane molecules, transmembrane and intracellular domains. N-CAM (neural cell adhesion molecule). DCC (deleted in colorectal cancer). MAG (myelin associated glycoprotein). FGF-R (fibroblast growth factor receptor). Ig (immunoglobulin). GPI (glycophosphatidylinositol). F3 (contactin).

1.2 Structure and functional properties of Ig CAMs

1.2.1 NCAM

The cell adhesion molecule NCAM is ubiquitously expressed and can be found on nearly all postmitotic neurons, on Schwann cells, oligodendrocytes, astrocytes and muscle cells (Moore and Walsh, 1986; Neugebauer et al., 1988; Seilheimer and Schachner, 1988).

NCAM exists in three major membrane-bound isoforms which are produced by alternative splicing. These three major membrane-bound

isoforms are named after their approximate molecular weight: NCAM-120, NCAM-140 and NCAM-180. The extracellular part of all NCAM isoforms consists of five Ig and two Fn type III modules. Transmembrane isoforms (NCAM 180 and 140 kDa), contain additionally transmembrane and intracellular domains whereas NCAM 120 is anchored to the membrane via glycosylphosphatidylinositol (Hemperly et al., 1986; Fig. 2).

An important impact on functional properties of NCAM is its modification. One type of NCAM modification is the attachment of a 2.8-polysialic polymer to the fifth Ig-like domain. This process is calcium dependent and is regulated by two different enzymes, designated ST8SiaII/STX and ST8SiaIV/PST (Ong et al., 1998). By attenuating cell-cell contacts, the polysialylated form of PSA-NCAM remains expressed in adult brain regions exhibiting a permanent capacity for structural and synaptic plasticity, including the olfactory bulb, the hippocampus and the pituitary gland (Bonfanti et al., 1992; Gubkina et al., 2001). A second type of NCAM modification is the alternative spliced exon (VASE) which is a 10 amino acid sequence being inserted into the fourth Ig-like domain. The VASE insert has been found in every major isoform of NCAM (Small and Akeson, 1990). Its presence correlates with a decreased capacity of NCAM to promote neurite outgrowth without, however, affecting adhesive properties (Lahrtz et al., 1997). At the beginning of neural development, only less than 3% of the NCAM transcripts contain this exon. With later developmental progress maintenance of fasciculation and stabilisation of synaptic contacts are of more importance, which correlates with an increase of the amount of VASE transcripts increases up to 50% (Small et al., 1988).

NCAM is involved in several processes like signal transduction, cell proliferation, neural cell migration, neurite outgrowth, axon fasciculation, synaptic remodelling and regeneration (Bronner-Fraser et al., 1992; Doherty and Walsh, 1992; Jørgensen, 1995; Sporns et al., 1995; Fields and Itoh, 1996;Walsh and Doherty 1996; Cremer et al., 1997; Schachner, 1997; Niethammer et al., 2002; Mannes and Schachner 2007).

Furthermore NCAM regulates neurite elongation via its interaction with RPTPalpha (Bodrikov et al., 2005) and NCAM is important for synapse formation and stabilisation of neuron-neuron contacts (Sytnyk et al., 2002, 2004). NCAM and its polysialylated form - PSA-NCAM plays an important role in induction of long-term potentiation (LTP) and long-term depression (LTD) forms of synaptic plasticity. Ablation of NCAM severely reduces LTP and LTD in NCAM deficient (-/-) mice (Muller et al., 1996; Bukalo et al., 2004). Furthermore NCAM influences long-term memory formation and behaviour (Rose, 1996; Welzl and Stork, 2003) and in the regulation of emotional responses (Conboy et al., 2008).

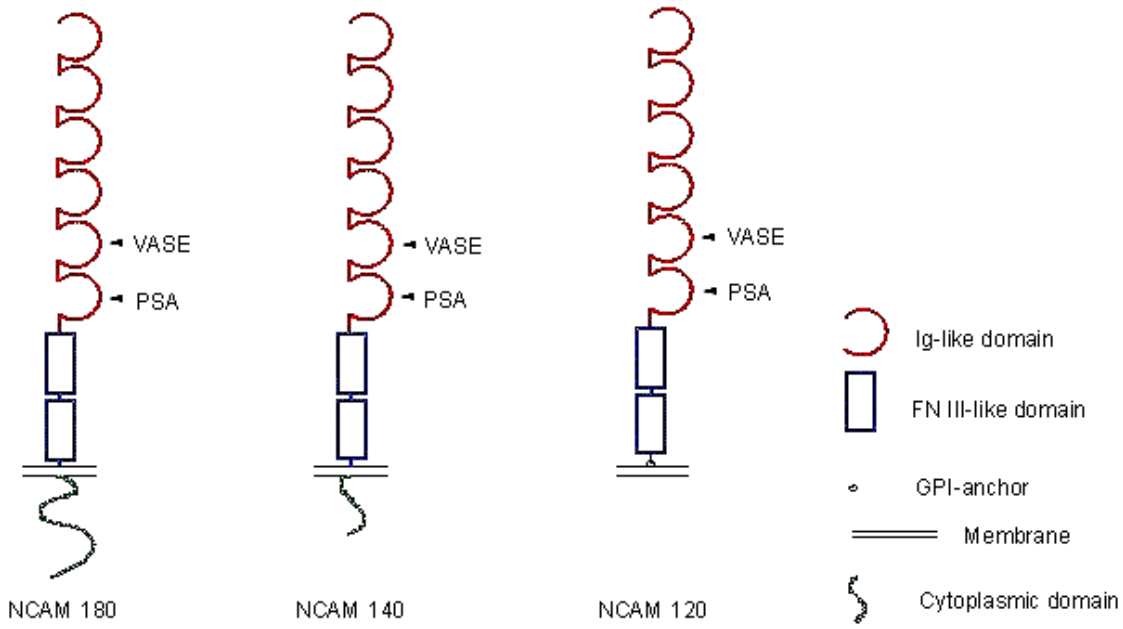

Figure 2. Modular structure of the major isoforms of NCAM. NCAM (neural cell adhesion molecule) consists of five Ig and two fibronectin type III (Fn III- like domain). NCAM 180 and NCAM 140 contain additionally transmembrane. NCAM 120 is anchored to the membrane via glycosylphosphatidylinositol (GPI-anchor). PSA (polysialic acid). VASE (variable alternative spliced exon). Arrowheads indicate the attachment site of PSA and the insertion site of VASE.

1.2.1.1 Phenotype of NCAM-/- mice

It is documented that NCAM 180-/- mice have deficits in neural migration (Wood et al., 1998). The olfactory bulbs of NCAM-/- mice are reduced in size as a result of disturbed cell migration. Later disorganised structure of pyramidal cell layer, reduction in fasciculation and number of mossy fibre bundles were found in the CA3 hippocampal area of NCAM-/- mice (Cremer et al., 1994).

NCAM-/- mice show abnormalities in function of mature synapses. Electrophysiological parameters characterising some forms of synaptic plasticity are impaired in NCAM-/- mice: LTP and LTD induction are severely inhibited in CA1 stratum radiatum of NCAM-/- mice (Bukalo et al., 2004). In addition the lack of NCAM is accompanied by reduced amounts of spectrin, NMDA receptors and CAMkinaseII at synapses (Sytnyk et al., 2006).

Regarding behaviour phenotype previous data revealed that NCAM-/- mice show deficits in spatial learning and exploratory behaviour and a strong increase in intermale aggression. It is also notable that NCAM-/- mice are more aggressive and anxious than wild type mice (Stork et al., 1997; Cremer et al., 2000). Anxiety-related behavioural alterations in NCAM-/- mice could be reduced by agonists of serotonin namely buspirone and 8-OH-DPAT (Stork et al., 1999). This finding indicates anxiety-related behavioural abnormalities to dysregulations of the serotoninergic system.

Recently, a study by Aonurm-Helm et al. (2008) showed that NCAM deficiency in mice resulted in depression-like behaviour by performing an increased freezing time in the tail-suspension test and a reduced preference for sucrose consumption in the sucrose preference test.

1.2.2 L1

L1 was one of the first isolated and characterised cell adhesion molecule (Rathjen and Schachner, 1984). The gene coding for L1 is located on the X-chromosome (Kohl et al., 1992; Kallunki et al.,1997).The cytoplasmatic domain of L1 shows a remarkable evolutionary conservation, being identical in human, rat and mouse. The presence of homologues across diverse species and the high degree of conservation in the course of evolution speaks for a key role of L1.

The neural cell adhesion molecule L1 is a transmembrane molecule of 200-220 kDa. Extracellular part of L1 consists of six Ig-like domains and five Fn type III repeats followed by a transmembrane region and a cytoplasmatic tail. L1 could be heavily glycosylated (Rathjen and Schachner, 1984) and is present in the central and peripheral nervous system but is also expressed in some non-neural tissue such as the crypt cells of the intestine (Thor et al., 1987), the epithelia of the kidney (Nolte et al., 1999) and T- and B-cells of the immune system (Ebeling et al., 1996). The upregulation of expression in tumour cells suggests its involvement in cancer (Meli et al., 1999). In the nervous system L1 is involved in processes like elongation and fasciculation of axons, neuronal survival, migration of neurons, adhesion between neurons and between neurons and Schwann cells, myelination in the peripheral nervous system, migration of neuronal cell bodies and synaptic plasticity (Lindner et al., 1983; Faissner et al., 1984; Rathjen and Schachner, 1984; Fischer et al., 1986; Lagenaur and Lemmon, 1987; Persohn and Schachner, 1987; Wong et al.,1996; Cohen et al., 1998; Dahme et al., 1997; Hulley et al., 1998; Chen et al., 1999). In addition, L1 has been implicated in axonal regeneration (Martini and Schachner, 1988) neuronal cell survival (Chen et al., 1999; Nishimune et al., 2005) and proliferation and differentiation of neurons (Dihné et al., 2003). Furthermore learning and memory formation (Rose, 1995; Venero et al., 2004) and the establishment of long-term potentiation in the hippocampus (Lüthi et al., 1996) are modulated by L1.

1.2.2.1 Phenotype of L1 deficient mice

L1 deficient (-/y) mice have similar abnormalites to those observed in humans with mutations in L1 gene, described as CRASH (Corpus callosum hypoplasia, mental Retardation, Adducted thumbs, Spastic paraplegia, Hydrocephalus) syndrome. There are defects in the development of the corticospinal tract and cerebellar vermis, hydrocephalus and impaired learning in humans and mice (Kamiguchi et al., 1998).

Studies in L1-/y mice suggest that L1 is also important for embryonic brain histogenesis, particularly in the development of axonal tracts. The L1-/y mice show a small but significant reduction in neural crest cell migration at early developmental stages (Brümmendorf et al., 1998; Anderson et al., 2006). L1 is a component of the Semaphorin3A (Sema3A), a repulsive axon guidance molecule, receptor complex. Ablation of L1 in mice may disrupt Sema3A signalling in the growth cone, leading to axonal guidance errors in the pathways of axonal guidance (Castellani et al., 2000). Moreover the cell adhesion molecule L1 is involved in the organization of dopaminergic neuronal cell groups in the mesencephalon and diencephalon but is altered in the distribution of dopaminergic neurons in the brain of L1-/y mice (Demyanenko et al., 2001). The study of Thelin and colleagues (2003) in L1-/y mice has shown decreased nociceptive heat sensitivity due to altered central processing of nociceptive withdrawal reflexes.

1.3 CAM knockout mice – their connection to neuropsychiatric disorders

1.3.1 NCAM-/- mice

A growing body of evidence implicates that NCAM dysregulations in NCAM-/-mice are involved in neuropsychiatric disorders such as

schizophrenia, major depression, bipolar disorder, anxiety disorder and Alzheimer disease (Vawter et al. 1998, Brennaman et al., 2008). Correlations of schizophrenia and hallmarks in NCAM-/- mice have been merged NCAM-180-/- mice display increased lateral ventricle size and reduced prepulse inhibition of startle response (Wood et al., 1998). Genetic deletion of NCAM-180 produces distinct defects in the central nervous system (Tomasiewicz et al., 1993) like reduction in size of the olfactory bulb and deficits in spatial learning (Cremer et al., 1994).

A study by Wakabayashi and colleagues (2008) found a reduction in the expression levels of NCAM-140 mRNA in patients with bipolar disorder in a current depressive state whereas L1 mRNA levels were increased in bipolar disorder patients in a current depressive state. Having analysed brains of schizophrenic patients, a reduction in polysialylated NCAM in the hippocampus (required for proper axon guidance) was observed (Barbeau et al., 1995) as well as increased concentrations of NCAM in the cerebrospinal fluid and of an extracellular fragment of NCAM in serum of patients with schizophrenia (Lyons et al., 1988).

It is also noteworthy that NCAM-/- mice express aggressive and anxious behaviour compared to wild-types (Storck et al., 1999).

1.3.2 L1-/y mice

L1 contributes to LTP (Lüthl et al., 1994) and the perturbation of L1 by specific antibodies leads to alteration in learning and memory (Scholey et al., 1995).

L1-/y mice are smaller than wild-type littermates. They show reduced sensitivity and were less sensitive to touch and pain. Their hind-limbs appear weak and uncoordinated, probably due to reduced corticospinal tract (Dahme et al., 1997). Depending on genetic background, the lateral ventricles were often enlarged (Dahme et al., 1997). Patients with mutations in close homologue of L1 (CHL1), located on human chromosome 3p26.1, have complex clinical syndromes

including mental retardation, neurological dysfunctions and hydrocephalus. Ablation of L1 gene in L1-/y mice results in similar phenotype including hypoplasia of the corticospinal tract, corpus callosum and ventricular enlargement (Dobson et al., 2001).

1.4 Chronic stress

1.4.1 Stress

Stress is a term that has become overused in modern life carrying a very negative and heavy notion. However, stress is a normal physiological and protective response to a hostile environment in which the organism tries to overcome threatening challenges by certain physiological adjustments. Regular stress response is essential for maintenance of homeostasis and survival. There are two components of stress response. One is the physiological way by heightened bodily arousal and second psychological by involving behaviour and emotions. Physiological aspects of stress were first described by Cannon (1915). Cannon introduced the term flight-or-fight to describe physiological mobilization of the organism for a quick response to danger (Cannon, 1915). Also Selye (1956) contributed to the development of biological aspects of the stress concept. He found that the fight-or-flight response was only the first in a series of reactions, which he called the general adaptation syndrome (GAS) (Selye, 1956). The GAS consists of three stages, namely alarm reaction, stage of resistance and stage of exhaustion. Physiological consequences of the stage of exhaustion could be caused by chronic stress. The reserves of the energy of the body are weakened and limited in its resistance because those regulatory mechanisms were established against short-term stress exposure. If the organism fails to repress stress-maladaptive responses restraint stress can lead to physical, physiological or psychological health problems. The chronic stress-induced physiological dysregulation is based

on neuroendocrine function, immune system, cardiovascular function and metabolic pathways which lead to declining health ranging from hypertension, atherosclerosis, the insulin-resistance-dyslipidemia syndrome, weakened immune system (Vanitallie 2002), hormonal imbalance, weight loss or weight gain and to psychiatric disorders like major depression and anxiety disorder.

1.4.2 Physiological responses to chronic stress

The immediate reaction to stress is the release of several hormones which direct the body for a physical peak performance, mainly: adrenaline, cortisol, thyroxin and testosterone. During stress responses, the liver releases sugar for energy, the heart rate increases and more blood is circulating to mobilise the muscle function. Parts of the body like digestion and sexual function which aren't needed urgently are turned off. Long-term stress inflicts damage to the cardiovascular circulation, metabolic and immune system as well as the structure and function of the brain.

Activation of the stress response system influences the hypothalamic-pituitary-adrenal axis, the sympathetic nervous system and the immune system and their interaction with each other. Important elements of the stress system affecting brain activity are the corticotropin-releasing hormone (CRH) and locus ceruleus-norepinephrine (LC/NE)-autonomic systems and their peripheral effectors, the hypothalamic-pituitary-adrenal (HPA) axis and the limbs of the autonomic system. These systems perpetuate with each other and interact with other neurotransmitter systems in the brain. The hypothalamic-pituitary-adrenal (HPA) axis is a sensitive physiological system which activation is triggered by chronic stress with the release of ACTH and glucocorticoids (Miller et al., 2002). A stressor initiates the release of corticotropin-releasing hormone (CRH) from the hypothalamus, which results in release of adrenocorticotropin hormone (ACTH) from the pituitary into general

circulation. ACTH then acts on the adrenal cortex followed by the release of a species-specific glucocorticoid into blood. Glucocorticoids act in a negative feedback fashion to limit the release of CRH.

The role of CRH and LC/NE system is to stimulate arousal and attention, as well as the mesocorticolimbic dopaminergic system, which is involved in anticipatory and reward phenomena, and the amygdala, which are responsible for the generation of fear (Chrousos, 2000). CRH inhibits also appetite and activates thermogenesis via the catecholaminergic system. In the central nervous system a HPA-axis dysregulation could damage the hippocampus which is necessary for long-term memory consolidation. Studies on effects of stress on the hippocampus revealed that glucocorticoid and CRH are the key molecules developing depressive disorder (Bao et al., 2007). Impairments of up- or downregulation of corticosteroids not only affect the molecular biology of the brain but also result in the devastating somatic diseases such as Cushing-syndrome, Addison- syndrome and atherosclerosis.

An organism faced with a severe external threat responds with the sympathoadrenal system (SAS) by a driven release of adrenal hormones. The general discharge of SAS with the secretion of catecholamines from the adrenal medulla, corticosteroids from the adrenal cortex and adrenocorticotropin from the anterior pituitary are adaptive hormonal responses to acute stress (Axelrod et. al., 1984). These hormones can trigger the body to react with the fight-or-flight response (Cannon, 1915) by giving the body a burst of energy and strength while increasing the heart rate and lipolysis, piloerection, papillary dilatation and an increase of the sphincter tone of the bowel and bladder. In the central nervous system catecholamine seem to play a critical role in level of alertness, vigilance, orientation, selective attention, memory and cardiovascular responses to life-threatening stimuli, all components of regulating anxiety.

Stress hormones (glucocorticoids and catecholamines) also act on the immune system. An activated stress response system protects the organism from systemic "overshooting" with T helper lymphocyte 1

(Th1)/proinflammatory cytokines. Glucocorticoids and catecholamines inhibit IL-12, TNF-alpha, and INF-gamma and upregulate IL-10, IL-4, and TGF-beta production. But hyperactive or hypoactive stress response together with a dysfunctional neuroendocrine-immune system may contribute to immune-related diseases such as rheumatoid arthritis (Calcagni et al., 2006).

1.4.3 Chronic stress-related psychiatric disorders in humans

It has been observed that stress or depression can lead to atrophy and cell loss in the hippocampus (Duman 2004). Chronic stress-induced synapse instability and altered brain plasticity may play a pivotal role in the molecular neuropathology of schizophrenia, major depression, bipolar and anxiety disorders.

The diathesis–stress model explains behaviour as both a result of biological and genetic factors and life experiences. According to this model genetic predisposition in combination with environmental stress produces or exacerbates symptoms of major psychiatric disorders (Zubin et al., 1977). There have been many studies on the causal association between stressful life events and the onset of depressive episodes or about the effects of chronic stress on recurrence of depressive episodes. There is a general consensus that depressed patients experience more stressful life events before major depressive episodes than their control group.

Neurobiological consequences of chronic stress correspondingly lead to affective disorders involving structural, functional and molecular alterations in several areas of the brain. Chronic stress is furthermore associated with the pathogenesis of schizophrenia. Currently schizophrenia is considered to be both inheritably and environmentally regulated. The two-hit hypothesis for schizophrenia postulates that inherited abnormal early neurodevelopment followed by stress-induced impairments in postnatal maturation of the brain contribute to the development of this disease. This allows to explain high (50% in

homozygous twins) but not absolute hereditary occurrence of schizophrenia (Lewis and Levitt 2002; McGrath et al., 2003). A possible role of stress-induced alterations in neuroactive steroid metabolism is widely discussed in the pathology of schizophrenia and major depression. Cortisol is involved in the psychopathology of depression and is being associated with hyperactivity of the HPA-axis (Pariante, 2003). Besides the relevance of neuroactive steroids and the HPA-axis on stress-related disorders there are studies which illustrate that chronic stress enhances the response of mesocortical dopamine neurons which play a key role in the expression and exacerbation of schizophrenic symptoms (Finaly and Zigmond, 1997). Furthermore neuronal cell adhesion molecules are linked to schizophrenia due to abnormal expression of NCAM on human chromosome 11q23 and mutation in L1 at Xq28, known as L1 syndrome (Panicker et al., 2003).

Stress has also an effect on addiction to drug abuse. It has been shown that in addition to a dysregulation of the dopamine system also the hormonal brain stress system via an impaired HPA-axis plays an important role on the development of addiction. For instance, brains of addicted people respond very differently to stress than those of individuals who are not addicted (Cleck and Blendy, 2008). This could result in development of bipolar disorder in patients that are genetically more vulnerable to subsequent environmental stressors, episodes and drug abuse (Kessler, 1997; Paykel, 2003; Kapczinski et al., 2008).

1.4.4 Alterations in the expression of cell adhesion molecules NCAM and L1 in psychiatric disorders

Cell adhesion molecules NCAM and L1 are suggested to be involved in neuropsychiatric disease due to their diverse contribution to neuronal functions ranging from cell migration, axonal and dendritic projection to synaptic formation and plasticity (Schmid and Maness, 2008; Wakabayashi et al., 2008).

Many studies show diverse direct and indirect evidence that NCAM and L1 are involved in stress-related disorders. Thus mutations in NCAM and L1 genes are associated with bipolar disorder and schizophrenia (Webster et al., 1999; Atz et al., 2007). The number of hilar PSA-NCAM-immunoreactive cells in the hippocampal brain region of schizophrenia patients is reduced (Barbeau et. al, 1995). Increased levels of soluble isoforms NCAM are observed in cerebrospinal fluid of patients with mood disorder suggesting an elevated shedding of NCAM from neuronal plasma membrane (Poltorak et. al., 1996). The expression of L1 mRNA is, on the contrary, increased in a current depressive state of patients with bipolar disorder (Wakabayashi et al., 2008).

Several lines of evidence indicate that chronic stress modulates the expression of the neural cell adhesion molecule, its polysialylation, and L1. After a 21-day restraint stress treatment the hippocampal mRNA expression and protein levels of NCAM were decreased and L1 increased (Sandi et al., 2001; Touyarot and Sandi, 2002; Venero et al, 2002; Sandi et al., 2004). Since NCAM is involved in the stabilization of synaptic contacts its chronic stress-induced reduction results in structural alterations, including atrophy of apical dendrite in CA3 pyramidal cells and ultrastructural changes in mossy fiber terminals (Magarinos and McEwen, 1995 a+b; McEwen and Magarinos, 1997).

Although these studies demonstrate a link of cell adhesion molecules NCAM and L1 between stress and stress-related disorders, the used animal chronic stress paradigms apply excessively strong physical stressors or intravenous corticosterone injection that do not fully recapitulate the pressure of emotional stress human beings experience in their everyday life (non-traumatic stress conditions). Therefore we created a chronic unpredictable mild stress protocol and investigated its molecular, physiological and behavioural long-term effects on C57BL/6J adult male mice.

II AIMS AND EXPERIMENTAL DESIGN OF THE STUDY

The aims of this study were to:

1) create a chronic unpredictable mild stress (CUMS) protocol that would resemble non-traumatic stress conditions in human beings;

2) investigate the long-term versus the short-term molecular and behavioural effects induced by CUMS in mice;

3) investigate whether these changes would correlate with a dysregulation of the cell adhesion molecules NCAM and L1 in the hippocampus.

In order to have an experimental animal model that approaches the environmental and psychosocial stressors in humans we have designed a CUMS protocol. Possible behavioural responses due to CUMS and its long-term effects have been tested using tests implicating anxiety and depression-like behaviour in mice. Furthermore, we monitored body weight and weight of several organs known to be possibly affected by stress.

Two projects were designed in this study to test the short-term and long-term effects of chronic unpredictable mild stress on C57BL/6J adult male mice. In the first project we analysed the short-term effects of chronic unpredictable mild stress on molecular, behavioural and physiological parameters. In the second project our main interest was to study the long-term effects of chronic unpredictable mild stress on depression-like behavior and anxiety, physiological and molecular changes in chronic stressed mice in comparison to the results of the short-term effects in the first project. Both projects consist of the same procedures before having started the CUMS protocol but used the identical chronic unpredictable mild stress protocol, too. The projects differ from the time points when decapitation and behavioural tests on depression-like and anxious behaviour in mice were performed and their decapitation which were dependent on the termination of the projects (Fig. 3). The first project ended 2 days after termination of chronic unpredictable mild stress

whereas the second project terminated 14 days after omission of chronic stress. Before starting the chronic unpredictable mild stress protocol mice were characterised for their body weight, novelty-induced behavior and anxiety and then were subdivided into a stress and control group in order that the stress group expressed the same behavior as the control group before starting the chronic unpredictable mild stress protocol. The stress group had been isolated for 14 days before starting the chronic unpredictable mild stress to test the effect of isolation on body weight in C57BL/6J. Mice of the stress group were exposed to different socio-environmental stressors on a daily basis. These stressors followed an unpredictable order. The control mice were left undisturbed in groups of 4 to 5 mice. Body weights were recorded periodically. The tests on depression-like behavior (tail suspension test) and anxiety (elevated-plus maze test) in C57BL/6J mice were done one day before starting the CUMS protocol and two days after termination of chronic unpredictable mild stress (in project 1) or one day before the start of chronic stress exposure and nine to 14 days after termination of chronic unpredictable mild stress (in project 2). Mice which belonged to the first project investigating the short-term effects of chronic unpredictable mild stress were decapitated 2 days after termination of chronic stress. To test the long-term effects of chronic unpredictable mild stress mice were killed 14 days after termination of chronic stress (project 2).

Then, to study the possible involvement of the cell adhesion molecules NCAM and L1 in the regulation of cellular and behavioural chronic unpredictable mild stress response induced changes, we measured the hippocampal expression of NCAM and L1 mRNA and of mRNA for corticosteroid receptors MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) by real-time RT-PCR in C57BL/6J adult male mice. In addition blood samples were taken to measure the effect of our CUMS protocol on the HPA-axis by analysing the plasma corticosterone concentration with the I125-Radioimmunoassay (RIA). Furthermore

adrenal glands, spleens, testes and preputial glands of C57BL/6J adult male mice were collected after the mice were decapitated to evaluate the changes of their weight after chronic stress.

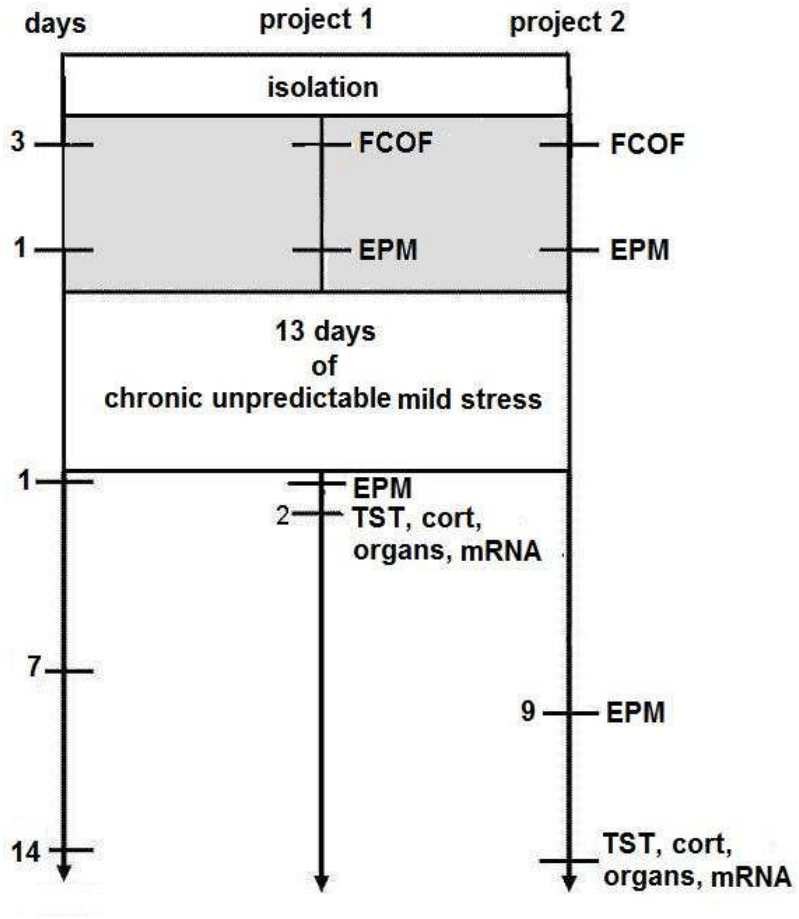

Figure 3. The study design of the projects to investigate the sort-term (project 1) and the long-term effects (project 2) on behavioural, physiological and molecular changes of chronic unpredictable mild stress on C57BL/6J adult male mice. cort (corticosterone); EPM (elevated-plus maze test); FCOF (free choice open field); hippocampal mRNA expression of NCAM, L1, GR and MR; TST (tail suspension test); grey box: the days before starting the chronic unpredictable mild stress protocol.

III MATERIALS AND METHODS

3.1 Animals and husbandry

The experiments belonging to the short- (project 1) and long-term (project 2) effects of chronic unpredictable mild stress (CUMS) were performed on 70 C57BL/6J male mice with the age of 15 weeks. Before starting the CUMS, mice were ear marked and their body weight (after arrival mice in the first project weighted around 32, 22 ± 1, 07 g and the mice of the second project weighted 30, 27 ± 0, 62 g) was measured. To ensure that the mice of the stress and control group express the same behaviour before starting the CUMS protocol their anxiety trait were characterised in the free-choice open field (FCOF) and elevated-plus maze test (EPM). Behavioural experiments were always conducted between 10.00 am and 5 pm. After the stress sessions, the stressed mice were returned to their home cages. During the period of the chronic unpredictable mild stress, control animals were left undisturbed in groups of 4 or 5 in their home cages. After the termination of the CUMS in the second project the stress group was left 14 days undisturbed while the mice of the control group were isolated on purpose to test the sucrose consumption (a test carried out on anhedonia) in the control and stress group after chronic unpredictable mild stress procedure. After five days the test on anhedonia which was tested with water bottles containing 5% sucrose was stopped due to the imprecise experimental device.

Mice were decapitated for the collection of the hippocampi and several organs (preputial gland, adrenal glands, spleen and testes). Also blood was collected 0, 30 or 60 min after tail suspension.

Husbandry

Mice were transferred from the central breeding facility to the experimental animal facility with an inverted 12:12 light/dark cycle (light off at 7:00, 21 ± 1°C, 40-50% humidity, food and water ad libitum). All

behavioural tests were performed during the dark cycle of the animals in a room next to the animal facility that was illuminated with dim red light. Tests which were part of the CUMS (rat room and 24 hours light, 24 and 15 hours exposure to rat) were performed overnight and the other least 2 hours after light offset and 2 hours before light onset, respectively but on different times due to unpredictability. The experimental materials were cleaned with soap, water and ethanol (70%) after each contact with an animal.

3.2 Chronic unpredictable mild stress (CUMS) protocol

day	starting time	socio-environmental stressors
1	9.30 am	cold water (forced swimming test)
2	10.30 am	rat room
3	11.00 am	24 hours light
4	2.00 pm	20 min intruder and resident
5	9.00 am	rat exposure
6	2.00 pm	bright illumination
7	11.00 am	cold water (forced swimming test)
8	10.00 am	rat exposure
9	11.00 am	24 hours light
10	10.00 am	24 hours exposure to rats
11	11.00 am	20 min intruder and resident +24 hours exposure to rats
12	6.00 pm	15 hours exposure to rats
13	12.00 pm	cold water (forced swimming test)

Figure 4. Chronic unpredictable mild stress (CUMS) protocol

We aimed to design a chronic stress protocol inducing depression-like symptoms in C57BL/6J mice which are comparable to non-traumatic stressful life experiences in human beings. Our chronic unpredictable mild chronic stress protocol (Fig. 4) lasts 13 days in which the stressors were presented once a day on different time points during the chronic stress

period and varied in their sequences, following an unpredictable, random order. Instead of applying strong physical stressors (e.g. footshock, food and water deprivation and prolonged immobilization) our chronic unpredictable mild stress protocol designed for this study consists of milder forms of environmental stress. To imitate stressful (non-traumatic) social life events in humans where people experience competitive social interactions by subordination to a dominant person, we introduced social stress (predatory and intrusive stressors) on C57BL/6J adult male mice.

3.2.1 Social stress (predatory and intrusive stressors)

Social stress (20 min intruder and resident)

This paradigm was used as a stressor for social stress. The stressed mouse was exposed to a dominant aggressive mouse which was conditioned to be aggressive in prior battles with mice. The stressed mouse as an intruder fought for 20 min with a duration of 5 min with each aggressive mouse (resident).

Rat exposure

The odour of a rat should stress the mouse, in a predatory way. A mouse of the stress group was placed into a cage oppositely to a rat while both rodents were divided by a mesh (Fig. 5). At where the side stressed mouse was placed the bathing was made up of 50% rat bathing and 50% fresh bathing. The duration of the test was 20 min for each mouse. After every mouse the bathing was exchanged and the cage was cleaned with soap, water and ethanol (70%).

Figure 5. Rat exposure

24 hours exposure to rats

The smell of rats should stress mice in an aversive and predatory way. The home cages of the stress group were placed for 24 hours next to the cages of the rats. In order to intensify the odour of the rats two handful of rat bathing of the rats were placed into the home cages of the stressed mice.

15 hours exposure to rats

This psychosocial olfactory stressor had the same principle as the rat exposure and 24 hours exposure to rat. The only difference was that the duration of this stressor was 15 hours.

Rat room

The aversive odour and predatory presence of rats in the same room was used as a social stressor in the chronic unpredictable mild stress protocol. The home cages of the mice were placed next to the cages of rats for 24 hours.

3.2.2 Environmental stressors

Cold water (Forced swimming test)

Mice were placed individually into glass cylinders (height 19 cm, diameter 15 cm) containing 989.601 cm³ water, maintained at 25±0,4°C. The animals were left in the cylinder for 5 min. Swimming, minor swimming and immobility were measured. The mouse was judged to be immobile when it remained floating passively and minor swimming when performing motions with one, two or three paws. The cold water/forced swimming test was not only used as an environmental stressor but also to test the effect of chronic unpredictable mild stress on depression-like behaviour in stressed mice.

24 hours light

This environmental, physical stressor should disrupt the 12 hours light and 12 hours dark cycle administered in the animal facility. The stressed mouse was placed in a room for 24 hours under constant illumination of 47 lx.

Bright illumination

The principle of this stressor was similar to the “24 hours light”. The light intensity of this stressor was 2000 lx during a period of three hours.

3.3 Behavioural analyses

3.3.1 Free choice open-field

The free choice open-field (FCOF) was performed to characterise mice for their anxiety before starting the chronic stress protocol and then to subdivide mice into a stress and a control group in order to establish

that mice of the two groups expressed the same behaviour before starting the stress protocol.

This test assesses trait anxiety, i.e. the intrinsic anxiety of an individual that is not elicited by external stimuli (Griebel et al. 1993). Mice were single-housed in transparent Plexiglas cages (23 x 17 cm and 14 cm high) with a hole (diameter: 4 cm) at the bottom of one of the small walls of the cage occluded by a door. The cage with a mouse was placed next to a rectangular open field (75 x 90 cm) surrounded by 30 cm high walls. The centre of one of the two 75 cm long walls of the open field had a gap where the small side of the cage fitted in, allowing direct access of the mouse from its cage in the open field when the door occluding the hole in the cage was open. The maze was surrounded by a black curtain and illuminated with a vertical white bulb (the light density in the open field was 5 lx). The test started when the door was opened. After the mouse had recognized that the door was open, it was given a maximum of 600 s to enter the open field with four paws. The test was interrupted either when a mouse had entered the open field or at the end of the 600 s. The latency to enter the open field (emergence latency) was calculated as the time that a mouse needed to step with all four paws into the open field starting from the moment it had recognized that the door was open.

3.3.2 Elevated-plus maze

The arena was made of white PVC which had a shape of a plus sign elevated 75 cm above the ground. Its four arms were 30 cm long and 5 cm wide, connected by a 5 x 5 cm central platform. Two opposing arms were bordered by 15 cm high walls (closed arms), whereas the other two arms (open arms) were bordered by a 2 mm rim to provide some gripping surface (Fig. 6). The test was carried out in the dark and videotaped with an infrared camera. The mouse was gently placed in the centre facing one of the open arms and observed for 5 min then returned to its home cage. Fecal boli which are droppings were documented at the end of the test.

Exploratory drive / anxiety of the mouse was deduced from the following parameters analysed with the software “The Observer”: latency to enter the open arms and closed arms, latency to reach the edge of the open arms, number of entries into the arms (calculated when all four paws on the arm) and centre (calculated when two paws in the centre), time spent in closed arms, time spent in open arms and in the centre, total transitions (the number of transitions between the closed and open arms), rearing on wall and protected head dips (exploratory head movement over the side of the open arms with the snout pointing downwards with only the two front paws on one of the open arms while the rest of the body remains in one of the closed arms or centre) and unprotected head dipping (movement of the head as in protected head dips but with all four paws on one of the open arms).

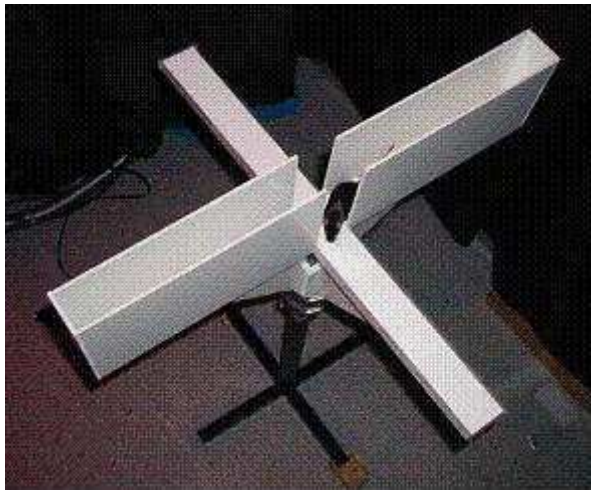

Figure 6. Elevated-plus-maze

3.3.3 Forced swimming test (cold water)

Mice were placed individually into glass cylinders (height 19 cm, diameter 15 cm) containing 989,601 cm³ water, maintained at 25°C±0,4°C. The animals were left in the cylinder for 5 min. During the test swimming; minor swimming and immobility were measured. The mouse was judged to be immobile when it remained floating passively and minor swimming when performing motions with one, two or three paws.

3.3.4 Tail suspension test

In the tail suspension test paradigm mice were tested for 6 min while hanging, with adhesive tape wrapped around its tail (1, 5–2 cm from tip), 80 cm above the floor. The duration of immobility, minor movements and locomotion were measured with “The Observer” programme. Mice were considered immobile when they hung passively and motionless, minor movement were movements with one, two or three paws whereas locomotion was defined as a movement with all limbs.

3.4 Collection of organs and tissue

Zero, 30 or 60 min after the tail suspension test which was carried out at the end of the projects all mice were decapitated to obtain their brains for the subsequent measurements of NCAM and L1 mRNA and of glucocorticoid (GR) and mineralocorticoid (MR) receptors mRNA. The hippocampi, adrenal glands, preputial glands, testes and spleen were resected and blood for the analysis of the corticosterone concentration had been collected.

3.5 I125-Radioimmunoassay (RIA) for the quantitative analysis of corticosterone in the plasma

The principle mechanism of the radioimmunoassay (RIA) is based on the concurrence between labeled corticosterone ^{125}I and unlabelled standard corticosterone respectively to be measured samples about limited binding sites of specific antibodies. The more unlabelled antigen the standards or to be analysed samples contain the less ^{125}I labeled antigen can be bound by the added antibody. After one step of incubation the amount of labeled complex of antibody and corticosterone is inversely

proportional to the amount of unlabelled corticosterone in the determined group. Low corticosterone concentration means more bound radioactivity and higher measurement and high concentration means less bound radioactivity and lower measurement.

From each plasma sample a precipitate was generated using a corticosterone ^{125}I radioimmunoassay kit for rats and mice (MP Biomedicals, Eschwege, Germany). Each precipitate was then diluted in 500µl 0.1 M NaOH, dissolved in liquid scintillation cocktail (Optiphase "HiSafe" 3, Perkin Elmer, Cologne, Germany) and counted in a liquid scintillation counter (Wallac 1409, Wallac-Perkin Elmer, Cologne, Germany) for 180 s. Values for each mouse were evaluated from duplicates of the original sample.

3.6 mRNA isolation

The right hippocampus which was frozen in -80°C had been disrupted and lysed with quiazol lysis reagent. The kit which was used is the Rneasy lipid tissue mini kit (50) from Quiagen. After the hippocampus sample was homogenized to cut genomic DNA and reduce viscosity of lysate. Then chloroform was added and the reagent was shaken vigorously. It was centrifuge after 2-3 min to separate phases. Aqueous phase was taken and ethanol was added to adjust binding conditions. The sample was then applied to the Rneasy spin column for adsorption of RNA to membrane. The contaminants with simple wash spins (Buffer RW1 and RPE) were removed. Finally, the elute was ready to use RNA in Rnase-free water.

3.7 Estimation of RNA and DNA content and purity of the samples

To estimate concentration and purity of the RNA or DNA (purified double stranded PCR product) aliquots of each sample were diluted 1:10 to 1:15 (depending on the expected amount of RNA or DNA) in RNAse/DNAse free water and measured in a UV spectrophotometer (APB, Freiburg,Germany) using the RNA or DNA quantification program. Since RNA and DNA molecules absorb UV light of a wavelength of 260 nm (A260), whereas (unwanted) proteins absorb strongest at 280nm (A280), the absorbance at 260 nm (A260) and 280 nm (A280), was measured against blank (RNAse/DNAse free water). Absorbance at 260 nm (A260) had to be higher than 0,1 but less than 1 optical density (OD) for reliable determinations. The OD at 260 nm (A260) was used to calculate the RNA and DNA concentration; absorption of 1 OD at 260 nm (A260) is equivalent to approximately 50 mg/ml DNA and 40 mg/ml RNA. The ratio of the readings at 260 nm and 280 nm (A260/A280) was used to estimate the purity of RNA and DNA. RNA preparations with a ratio of 1, 9 to 2, 1 and DNA samples with ratios of 1, 8 to 1, 9 were considered to be sufficiently pure for downstream applications.

3.8 Reverse Transcription (RT)

Reverse transcription (RT) was performed to synthesize first strand cDNA from RNA. After thawing the RNA samples on ice, equal amounts of RNA (1 µg – 3 µg) were diluted in a total volume of 10µl with RNAse/DNAse free water (Promega GmbH Mannheim, Germany). RT was carried out in a MJ Research PTC-200 DNA ENGINE Peltier Thermal Cycler (Biozym, Hessisch Oldendorf, Germany) with the SuperScript II Reverse Transcriptase kit (Invitrogen, Karlsruhe, Germany) using a hexamer primer (Metabion, Planegg, Martinsried, Germany) and a deoxynucleotide

solution mix containing 10 mM of each nucleotide (dNTP Mix, New England BioLabs, Frankfurt, Germany). To the mRNA has been added 1µl Hexamerprimer and 1µl dNTP. This mixture was heated to 65°C for 5 min and quickly chilled on ice. Afterwards 4µl buffer and 2 µl Dithiothreitol (DTT) were pipetted to the mixture. Then it was incubated at 25°C for 2 min. At the end 1µl SS II RT was added which incubated at 25°C for 10 min. Thereafter the mix content was incubated at 42°C for 42 min. The end of the thermal cycler reaction was inactivating the reaction by heating at 70° C for 15 min (Fig. 7).

Pipetting and cycler protocol:

Hexamer primer	1 µl		
dNTP Mix 10 mM each	1 µl	65°C	5 min
		4°C	5 min
5x first-strand buffer	4 µl		
0.1 M DTT	2 µl	25°C	2 min
SuperScript II RT	1 µl	25°C	10 min
		42°C	42 min
		70°C	15 min

Figure 7. Pipetting and cycler protocol of the reverse transcription (RT)

3.9 Real-time reverse transcription polymerase chain reaction (RT-PCR)

Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to measure levels of mRNA of NCAM L1, MR and GR in hippocampal tissue. Specific primers were designed based on published mouse mRNA sequences using the PrimerExpress 2.0 Software (Applied Biosystems, Darmstadt, Germany). The following primers were used: GR (forward 5'-CAG CAT GCC GCT ATC GAA A-3'; reverse 5'-CGC

GGC AGG AAC TAT TGT TTT-3'; Genebank accession number: NM_008173), MR (forward 5'-TGT CTC AGA CCT TGG AGC GTT C-3'; reverse 5'-TTG TTC GGA GTA GCA CCG GAA-3'; Genebank accession number: XM_983321), NCAM (forward 5´-TGC AGT TTG ATG AGC CAG-3´; reverse 5´CCT CCA TGT TGG CTT CTT-3´; Genebank accession number: NM_001081445), L1 (forward 5´-CCC ACC AAC AGC ATG ATT GAC-3´; reverse 5´AAT CCC TCA GCA ATG CAC TCC-3´ Genebank accession number: BC_056988) and for reference, hypoxanthine guanine phosphoribosyl transferase (HPRT; forward 5'-GTT CTT TGC TGA CCT GCT GGA-3'; reverse 5'-TCC CCC GTT GAC TGA TCA TT-3'; Genebank accession number: NM_013556). Oligonucleotides were synthesized by Metabion (Martinsried, Germany). Amplicons for all primers were sequence-analysed to verify specific amplification. Quantitative PCR was performed in a total reaction volume of 20 µl using a SYBR green master mix (qPCR Core Kit for SYBRGreenI, Eurogentec, Seraing, Belgium) with 1 µl (5 pmol) each of forward and reverse primer and 1 µl of the 1:5 to 1:10 diluted cDNA sample depending on the amount of RNA used for reverse transcription. Duplicates of the original sample were measured and analysed on an ABI 7900 HT sequencer (Applied Biosystems, Darmstadt, Germany). Amplification conditions were as follows: 45 cycles of 15 s at 95°C and 60 s at 60°C. Real-time PCR data analysis was performed using the comparative $2^{-\Delta\Delta C}$T method (Livak & Schmittgen 2001) with hypoxanthine guanine phosphoribosyl transferase (HPRT) as an endogenous reference. For graphical and statistical analysis, the mRNA level for each mouse was expressed as a percentage of the mean value of the control group which was set to 100% (Fig. 8).

Master-mix:		Amplification conditions:
10x Reaction buffer	2,0 µl	50°C 2 min
50mM MgCl	1,4 µl	95°C 10 min
5mM dNTPs	0,8 µl	
enzyme	0,1 µl	45 amplification cycles:
water	12,1 µl	95°C 15 s
SYBER Green	0,6 µl	60°C 60 s
primer	2,0 µl	
template/ sample	1,0 µl	95°C 5 s
		50°C 5 s

Figure 8. Master mix pipetting protocol and amplification conditions and cycles for the real-time reverse transcription polymerase chain reaction (RT-PCR)

3.10 Statistical analyses

Data were analysed either with the non-parametric Mann-Whitney test or the non-parametric Wilcoxon matched pairs test when appropriate. Data on forced swimming test on stressed animals were analysed with the three-way ANOVA for repeated measurements having “treatment” (stressed and control) as between group factor and “day” and “min” as within group factors. Part of data of the tail suspension test were analysed by two-way-Anova for repeated measurements having “treatment” as between group factor and “min” as within groups factor. Body weights were also analysed with two-way-Anova for repeated measurements having “treatment” as between group factor and “day” as within group’s factor. Values for the plasma corticosterone levels were analysed with two-way-ANOVA having as between group factors “treatment” and “time of killing”.

IV RESULTS

4.1 Project 1: Short-term effects of chronic unpredictable mild stress

4.1.1 Effects of chronic unpredictable mild stress on depression-like behaviour in C57BL/6J adult male mice in the forced swimming test

The forced swimming test (FST) is an animal model to screen antidepressant activity and is a most used tool for screening antidepressants (Petit-Demouliere et al., 2005). In our study the forced swimming test was used as a test to detect depression-like behaviour in the stress group and was applied as an environmental stressor (cold water) in the chronic unpredictable mild stress protocol.

A mouse displays “behavioural despair” in the FST on the assumption that the animal has given up hope of escaping when it is not swimming (immobile). Contrary to time spent in immobility is time spent in swimming, indicating a flight or fight reaction. Mice of the stress group underwent the forced swimming test at the 1st, 7th and 13th of the stress protocol. Stressed mice were left for 5 min in the water tank. Swimming and time spent in immobility had been measured. To test the effects of chronic unpredictable mild stress on depression-like behaviour we analysed whether time spent in immobility changed between the first day (day 1) and the last day (day 13) of the chronic stress protocol. After the 13th day of chronic stress exposure stressed mice stayed significantly longer immobile than in the 1st forced swimming test which is indicated by Wilcoxon matched pairs test ($p<0, 001$; Fig. 9 A). Also the time spent swimming was detected on day 1 and day 13 of chronic unpredictable mild stress. The Wilcoxon matched pairs test which is a paired non-parametric t-test showed that stressed mice swam significantly less after 13 days of chronic stress exposure in comparison to day 1 ($p< 0, 001$; Fig. 9 B).

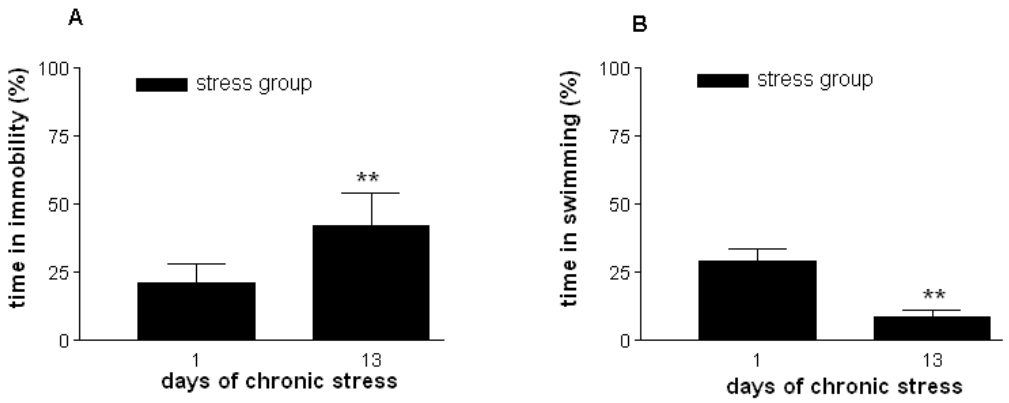

Figure 9. Developed depression-like behaviour of the stress group in the forced swimming test at day 1 and day 13 of chronic unpredictable mild stress. **A** Wilcoxon matched pairs test indicated a significant effect on time in immobility after 13 days of chronic stress compared to day 1 (**p< 0, 001). Graph **B** shows the reduced time in swimming in the stress group after 13 days of chronic stress exposure (**p< 0, 001).

4.1.2 Short-term effects of chronic unpredictable mild stress on anxiety in mice in the elevated-plus maze test

The elevated–plus maze is a mouse model of anxiety. The state of anxiety in mice, defined as anxiety that occurs temporarily as a reaction to an anxiogenic stimulus (Belzung and Griebel, 2001), which was evaluated in this study one day before starting the chronic unpredictable mild stress protocol to characterise the mice for their anxiety in order to see that mice expressed the same behaviour before starting the stress protocol. Furthermore the elevated-plus maze test was done one day after termination of chronic stress for the stress group and the control group. The time spent in open arms, open arm entries, number of total transitions (the number of transitions between the closed and open arms) and the number of rearing were analysed. There were no significant changes in the time spent in the open arms and in the percentage of open arm entries in both groups one day after chronic unpredictable mild stress than one day before starting chronic stress protocol (Fig. 10 A+B). No difference

was detected for the number of total transitions between the closed and open arms (Fig. 10 C). The amount of rearing, an indicator for exploratory drive, was significantly increased in the stress group one day after termination of chronic stress (p< 0, 05; Fig. 10 D).

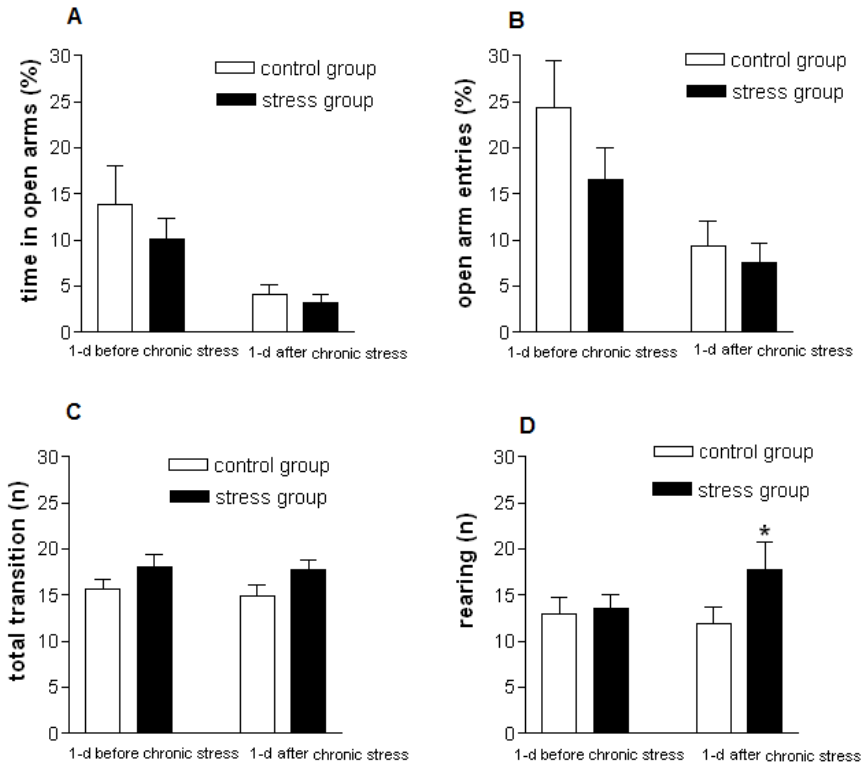

Figure 10. Elevated-plus maze test. Mice were tested in the elevated-plus maze 1 day before chronic unpredictable mild stress exposure and 1 day after chronic stress. **A** Percentage of time in the open arms. **B** Percentage of open arm entries. **C** Percentage of total transitions between closed and open arms. **D** Number of rearing bouts. Mann Whitney test (* p< 0, 05).

4.1.3 Short-term effects of chronic unpredictable mild stress on depression-like behaviour in mice in the tail suspension test

Similarly to the forced swimming test, the tail suspension test is used as an animal model for assessing antidepressant-like activity in mice. Antidepressant-like activity was tested in the control and stress group after chronic unpredictable mild stress treatment. Aside screening antidepressant-like activity in mice the test was also installed as acute stress to promote the corticosterone stress response.

The development of an immobile posture in inescapable stress of being suspended by their tail indicated depression-like behaviour in mice in the tail suspension test. To test if chronic stressed mice display depression-like behaviour, the stress and control group were evaluated in the tail suspension 2 days after termination of chronic unpredictable mild stress. Chronic stressed mice spent in the tail suspension test 2 days after termination of stress a significant time in immobility comparing with the control group ($p< 0, 01$; Fig. 11 A). Two-way ANOVA for repeated measures showed a significant effect of group ($p< 0, 05$), a significant effect of minute ($p< 0, 001$) and no effect of the interaction between group and minute ($p> 0, 05$). Post-hoc analysis indicated a significant difference between the control and stress group during the first minute of the test ($p< 0, 01$; Fig. 11 B).

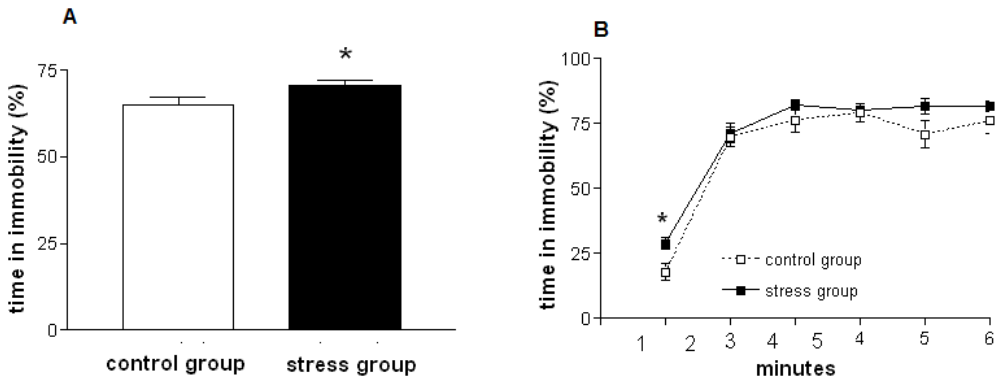

Figure 11. Depression-like behaviour in the tail suspension test 2 days after chronic unpredictable mild stress. **A** Time in immobility in percentage (*p<0, 01). **B** Time in immobility in percentage of stress (n= 18) and control (n= 12) group in minutes. There was a significant difference between the control and stress group during the first min of the test in the Post-hoc analysis (*p< 0, 01).

4.1.4 Short-term effects of chronic unpredictable mild stress on body weight in C57BL/6J adult male mice

Reduction of body weight is based on the assumption that mice under stressful conditions will lose body weight linked to the stress-induced hormonal and metabolic regulations. Body weight of the stress and control group was measured in the days of isolation, during chronic stress and 2 days after exposure to chronic unpredictable mild stress.

4.1.4.1 Body weight during isolation

Body weight was measured on the first day of isolation and on the 6th day of isolation. Two-way ANOVA for repeated measures indicated a significant effect between the stress and control group at the first day of isolation (p< 0, 01; Fig. 12 A).

4.1.4.2 Body weight during chronic unpredictable mild stress

Body weight was taken on the 1st, 7th and 13th day of chronic unpredictable mild stress protocol to analyse the immediate influence on body weight in chronic stressed mice. During the chronic stress protocol the control group was still kept in groups of 4. Two-way ANOVA for repeated measures showed a significant effect of days ($p < 0,05$; Fig. 12 B). Post-hoc analysis indicated a significant difference between stressed and control group at the 7th day of chronic unpredictable mild stress protocol ($p < 0,05$) and a significant difference between both group at the 13th day of chronic stress ($p < 0,05$).

4.1.4.3 Body weight 2 days after chronic unpredictable mild stress

The body weight was measured additionally 2 days after chronic stress. At this time point the control group was immediately isolated after termination of chronic stress. Two-way ANOVA for repeated measures showed a significant effect of days ($p < 0,001$; Fig. 12 C). Post-hoc analysis indicated a significant difference between stress and control group 2 days after termination of chronic stress ($p < 0,001$).

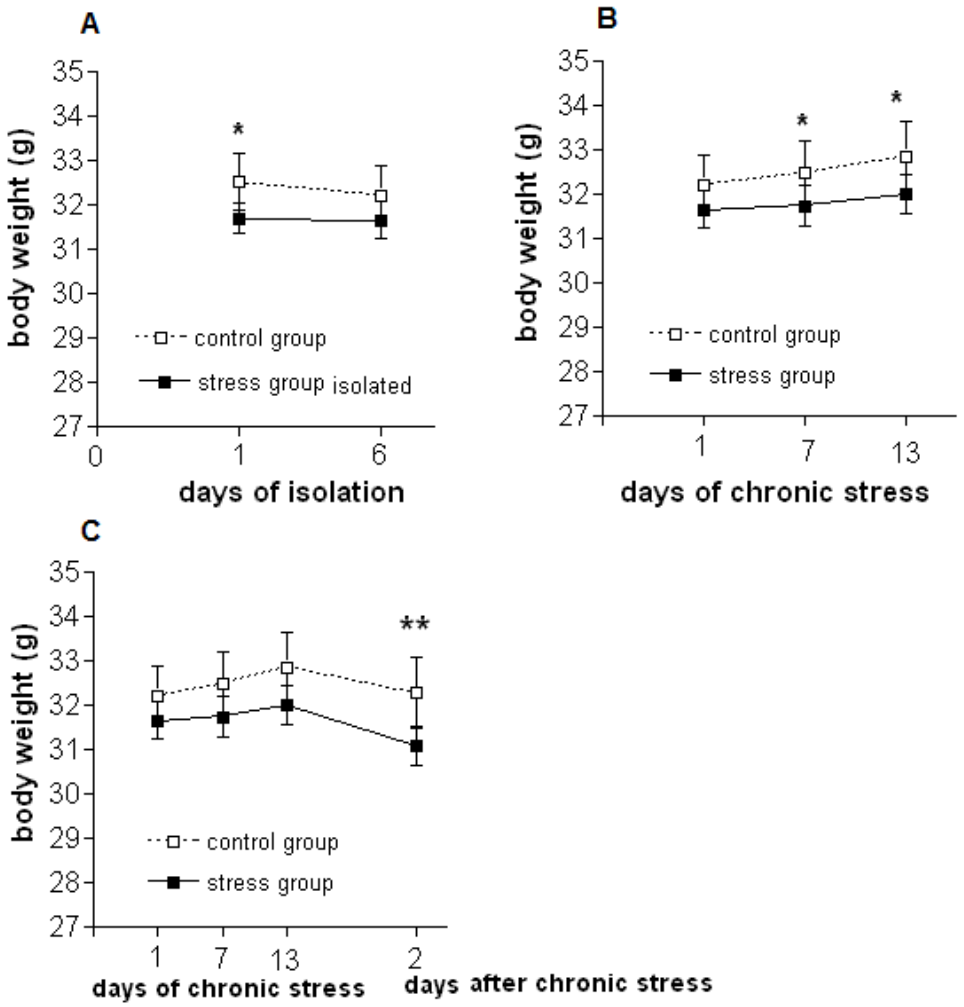

Figure 12. A Changes of body weight in the control (n= 12) and the stress group (n=18) during isolation (*p< 0. 01), **B** in the days of chronic stress exposure (*p< 0. 05) and **C** 2 days after chronic unpredictable mild stress (** p< 0, 001).

4.1.5 Short-term effects of chronic unpredictable mild stress on weight of organs in C57BL/6J adult male mice

Preputial gland. In rodents the preputial glands are one of the major sources of pheromones and important for urine marking. These chemosignaling organs are known to elicit specific behavioural and physiological effects in their conspecifics. Social stress can alter both the behaviour and hormonal status of rodents and are thus typically reduced in submissive male mice. We examined if the predatory and intrusive

stressors of our chronic unpredictable mild stress protocol altered significantly the weight of preputial glands in C57BL/6J male mice.

Adrenal glands. Corticosterone, a stress hormone which is found in species like rodents amphibians, reptiles, and birds is secreted in the in the adrenal cortex. The adrenal glands, the hypothalamus and the pituitary gland are elements of the HPA-axis, a neuroendocrine system that responds to stress. Based on previous observations that chronic stress induced hypertrophy of adrenal glands in mice we tested whether our newly degenerated chronic stress paradigm changes the weight of this organ.

Testes. Chronic psychological and social stresses can impair reproductive hormone secretion in a variety of non human primate species (Cameron, 1997). This impaired reproductive hormone secretion is due to a complete suppression of fertility and reproductive behaviour. The functions of the testes are the testosterone production and sperm cells production. Therefore the testis is an important organ for male fertility. The aim of the measurements was to test if this chronic unpredictable mild stress altered the weight of testes in the stress group.

Spleen. The spleen is part of the unspecific and specific defence of the immune system by storing macrophages and lymphocytes. The immune system is particularly sensitive to stress and an imbalance of neuroimmunomodulation can be induced by stress (Galinowski, 1993). It is tempting to hypothesize that chronic stress-induced immunodepressive dysfunction may be linked with a change in weight of spleens in C57BL/6J adult male mice.

Preputial glands, adrenal glands, testes and spleens were taken 2 days after chronic stress to measure the short-term effects of chronic unpredictable mild stress on these organs.

The weight of the preputial glands was significantly reduced in the stress group compared to the control group ($p< 0, 05$; Fig. 13 A). The weight of adrenal glands of the stressed mice was significantly enlarged 2

days after chronic stress (p< 0, 001; Fig. 13 B). Compared to the control group testes of the stressed mice were not affected in the short-term by the chronic stress treatment. Also the weight of spleens of both groups did not differ from each other significantly 2 days after chronic stress (Fig. 13 C+D).

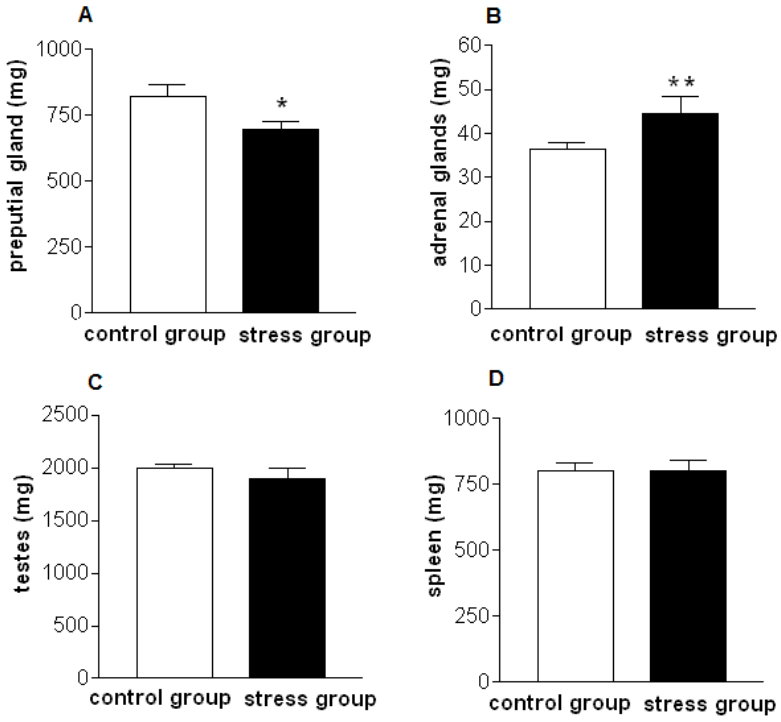

Figure 13. A The weight of the preputial gland was significantly decreased in the stress group (n=18) 2 days after chronic unpredictable mild stress (*p< 0, 05). **B** Adrenal glands of the stressed mice (n=18) were significantly heavier (**p<0,001). **C** No changes in weights of testes in stressed mice (n=18) during 13 days. **D** Weight of spleen of the control (n=12) and stress (n=18) group did not differ after chronic stress treatment.

4.1.6 Short-term effects of chronic unpredictable mild stress on the plasma corticosterone concentration in C57BL/6J adult male mice

The hypothalamic-pituitary-adrenal (HPA) axis is a main part of the neuroendocrine system which controls the response to stress. Corticotropin-releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus (PVN) induce adrenocorticotropic hormone

(ACTH) release from the pituitary, which subsequently causes corticosterone release from the adrenal cortex. The HPA-axis is a sensitive physiological system whose activation, with the consequent release of ACTH and glucocorticoids, is triggered by a wide range of physiological and psychosocial stressors. HPA-axis dysregulation is characterised by impaired circulating corticosterone concentrations and deregulated negative feedback inhibition which can be induced by chronic stress. The aim was to test if chronic unpredictable mild stress affects the concentrations of plasma as indication of an altered activity of the HPA-axis in mice.

Blood was collected from mice of the stress and control group 0, 30 and 60 min after the tail suspension test that served as an acute stressor to activate the HPA-axis in the mice 2 days after termination of chronic unpredictable mild stress. The two-way ANOVA having as between group factors “group” and “time of killing” indicated a significant effect of the time mice were killed ($p< 0, 01$; Fig. 14). Newman-Keuls post-hoc analyses indicated that increase of levels of corticosterone from 7 min up to 30 min are not significantly higher in the stress group compared with the control group 30 min after tail suspension ($p= 0, 5$).

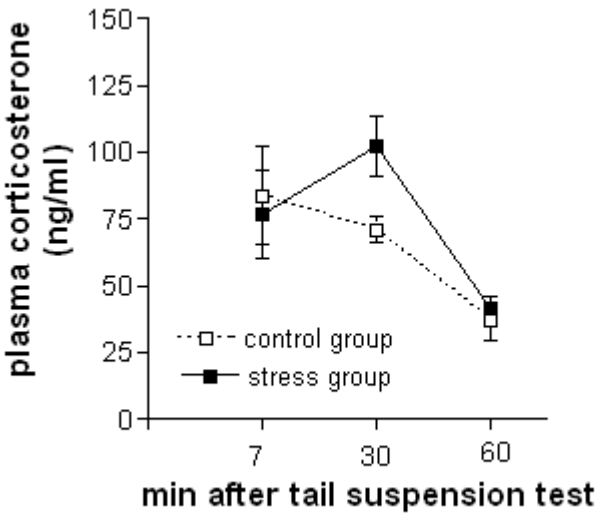

Figure 14. Plasma corticosterone concentration of stress and control group 2 days after termination of chronic unpredictable mild stress. Blood samples were collected 7, 30, 60 min after tail suspension test to measure stress-induced plasma corticosterone levels.

4.1.7 Short-term effects of chronic unpredictable mild stress on mRNA expression of cell adhesion molecules NCAM, L1 and corticosteroid receptors MR and GR in the hippocampus of C57BL/6J adult male mice

Chronic stress has been reported to induce deleterious effects on structure and function in the hippocampus by damage of synaptic plasticity and neuronal cell loss. The cell adhesion molecules NCAM and L1 as a mediator for synaptic plasticity has been suggested to play a role in the regulation of structure remodeling in the dentate gyrus after restraint stress exposure (Pham et al., 2003).

The aim of this study was to investigate both the short- and the long- term effects of the new chronic unpredictable mild stress in changes in mRNA expression of NCAM and L1 in the right hippocampus based on the method of real time reverse transcriptase polymerase chain reaction (RT-PCR).

In addition, we were interested to find out if corticosteroid receptors MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) are regulated in the hippocampus in response to chronic unpredictable mild stress as corticosteroid receptors are key mediators of the neuroendocrine response to stress and are widely represented in neurons in the hippocampus (de Kloet et al., 1998). It has been reported that chronic stress-induced dysregulation of expression of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) appear to make nervous tissue vulnerable to damage (Sousa et al., 2008).

RT-PCR of the right hippocampus has been carried out with the aim to analyse alterations in the mRNA expression level expression of NCAM, L1, GR and MR 2 days after termination (short-term effect) of chronic unpredictable mild stress.

The evaluation of the effect of chronic unpredictable mild stress in the short-term showed that NCAM mRNA expression in the hippocampus is significantly enhanced ($p < 0, 05$; Fig.15 A) whereas L1 mRNA in the hippocampus was not significantly changed in the stress group after two days after chronic stress (Fig. 15 B). On the one hand the hippocampal mRNA level of GR in the stress group was not affected by chronic unpredictable mild stress treatment while on the other hand the mRNA expression of MR was significantly increased compared to the control group ($p < 0, 05$; Fig. 15 C+D).

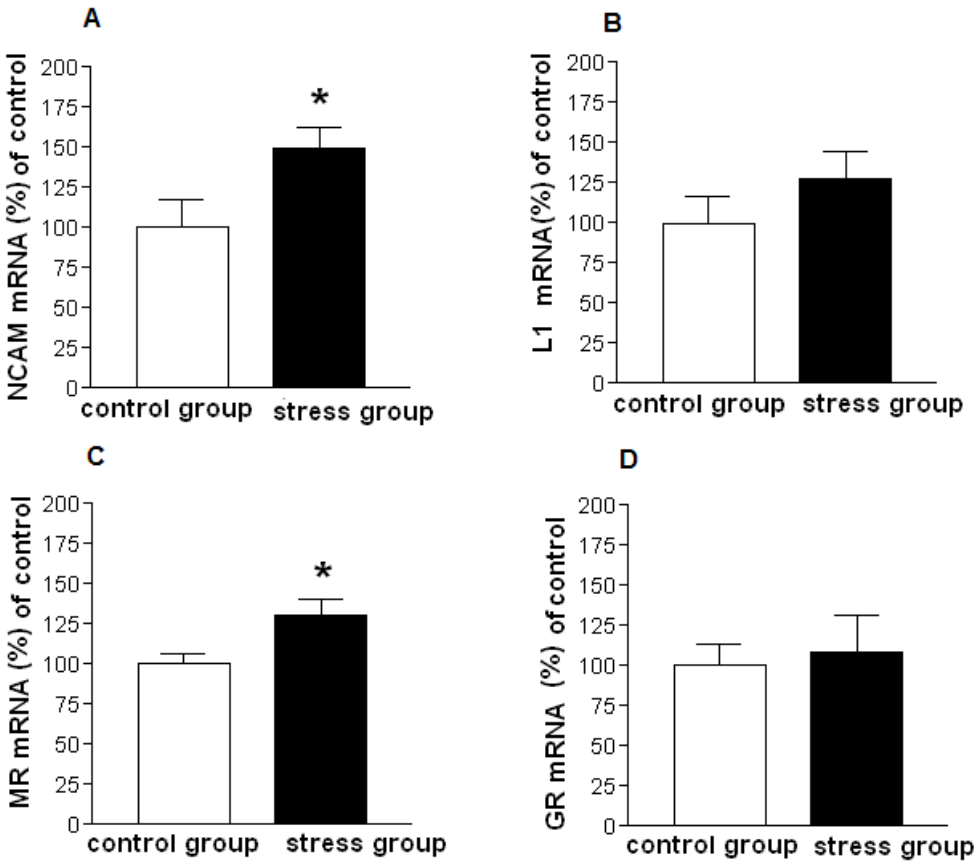

Figure 15. Hippocampal mRNA expression of **A** NCAM, **B** L1, **C** MR and **D** GR. Right hippocampi were removed 2 days after termination of chronic unpredictable mild stress and mRNA expression of NCAM, L1, MR and GR receptors were quantified with RT-PCR. **A**+**C** Chronic stress treatment increased both NCAM and MR expression *p< 0, 05 by Mann Whitney test whereas mRNA expressions of L1 **B** and GR **D** were not affected by chronic unpredictable mild stress.

4.2 Project 2: Long-term effects of chronic unpredictable mild stress

4.2.1 Effects of chronic unpredictable mild stress on depression-like behaviour in C57BL/6J adult male mice in the forced swimming test

In the second project, in which we studied the long-term effects of chronic unpredictable mild stress, exactly the same stress protocol was used. The cold water/forced swimming test, was on the one hand used as an environmental stressor for the stress group and on the other hand as a model to evaluate the effects on depression-like behaviour during exposure to chronic unpredictable mild stress. The forced swimming test was performed with the stress group on the 1st, 7th and 13th day of the chronic unpredictable mild stress protocol. In the forced swimming test chronic stressed mice were left in the water tank for 5 min. The time stressed mice spent in immobility significantly increased on the 13th day of chronic stress compared to the time spent in immobility on the 1st day of chronic unpredictable mild stress ($p< 0, 001$; Fig. 16 A). In addition chronic stressed mice swam after 13 days of chronic stress treatment less than on the 1st day of stress ($p< 0, 0001$; Fig 16 C). Two-way ANOVA for repeated measures having as within group factors “day” and “minute” showed a significant effect of day ($p< 0, 0001$, Fig. 16 B) and an effect of the interaction between day and minutes ($p< 0, 0001$). A post-hoc analysis indicated a significant difference in the first minute between day 1 and day 13 of the forced swimming test ($p< 0, 0001$). There was also a significant difference of time in immobility in the stress group in the second minute of the forced swimming test between day 1 and day 13 ($p< 0, 0001$, Fig. 16 B) and a significant difference on the third minute of the first and third tail suspension test ($p< 0, 01$).

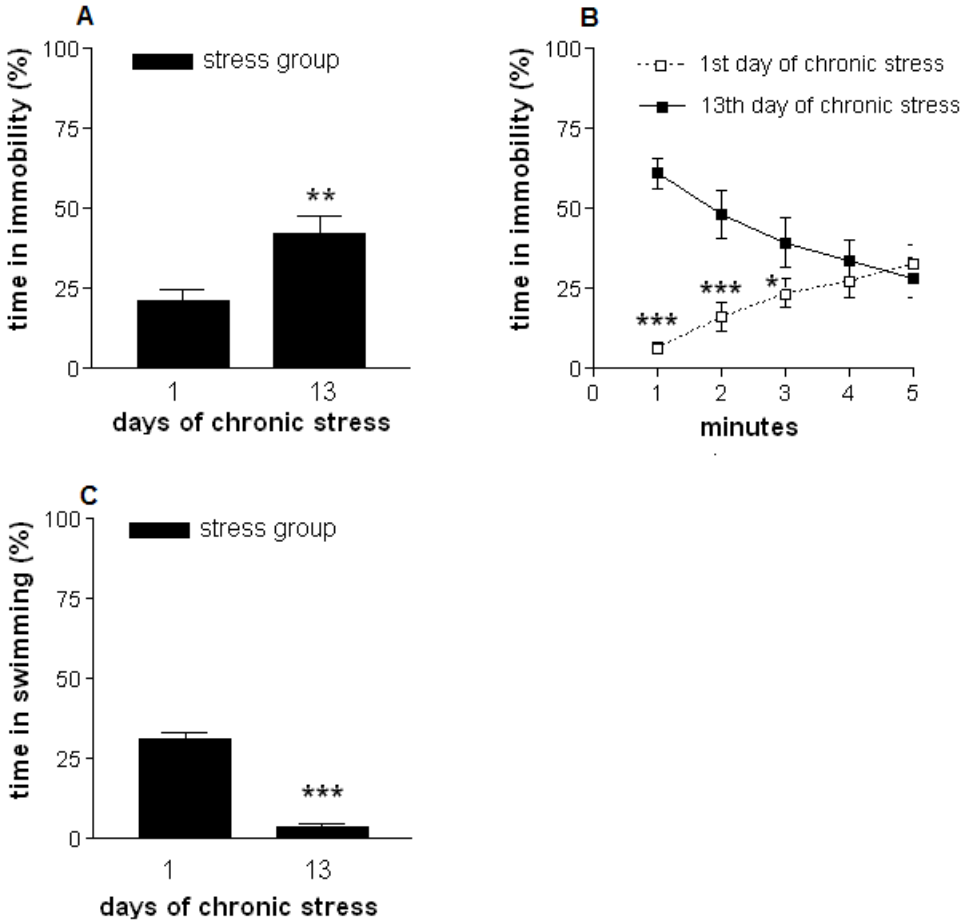

Figure 16. Depression-like behaviour of the stress group (n= 20) in the forced swimming test. **A** There was an increase in the percentage of time in immobility (**p< 0, 001) from the 1st day up to the 13th day of chronic unpredictable mild stress. Graph **B** shows 1 min intervals of 5 min recording of time in immobility compared between 1st day and 13th day of chronic unpredictable mild stress tested by post-hoc comparison with repeated measurements (***p< 0, 0001; *p< 0,01). **C** There was a significant reduction in Wilcoxon matched pairs test in the time in swimming in stressed mice after 13th days of chronic unpredictable mild stress (***p< 0, 0001).

4.2.2 Long-term effects of chronic unpredictable mild stress on anxiety in mice in the elevated-plus maze test

Besides investigating the long-term effects on depression-like behaviour we tested whether chronic unpredictable mild stress had a long-term effect on anxiety. The analysis with the Mann Whitney test revealed that the stress group stayed more often (p< 0, 05; Fig. 17 A) in the open arms nine

days after termination of chronic unpredictable mild stress protocol than the control group. Similarly, there were more open arm entries in percentage nine days after chronic stress in the stress group compared with the control group on the same time point (p< 0, 05; Fig. 17 B). The Mann Whitney test on the total transitions (the number of transitions between the closed and open arms) and the amount of rearing did not show any significant differences regarding an anxious behaviour between the stress and control group one day before and nine days after chronic unpredictable mild stress (Fig. 17 C + D).

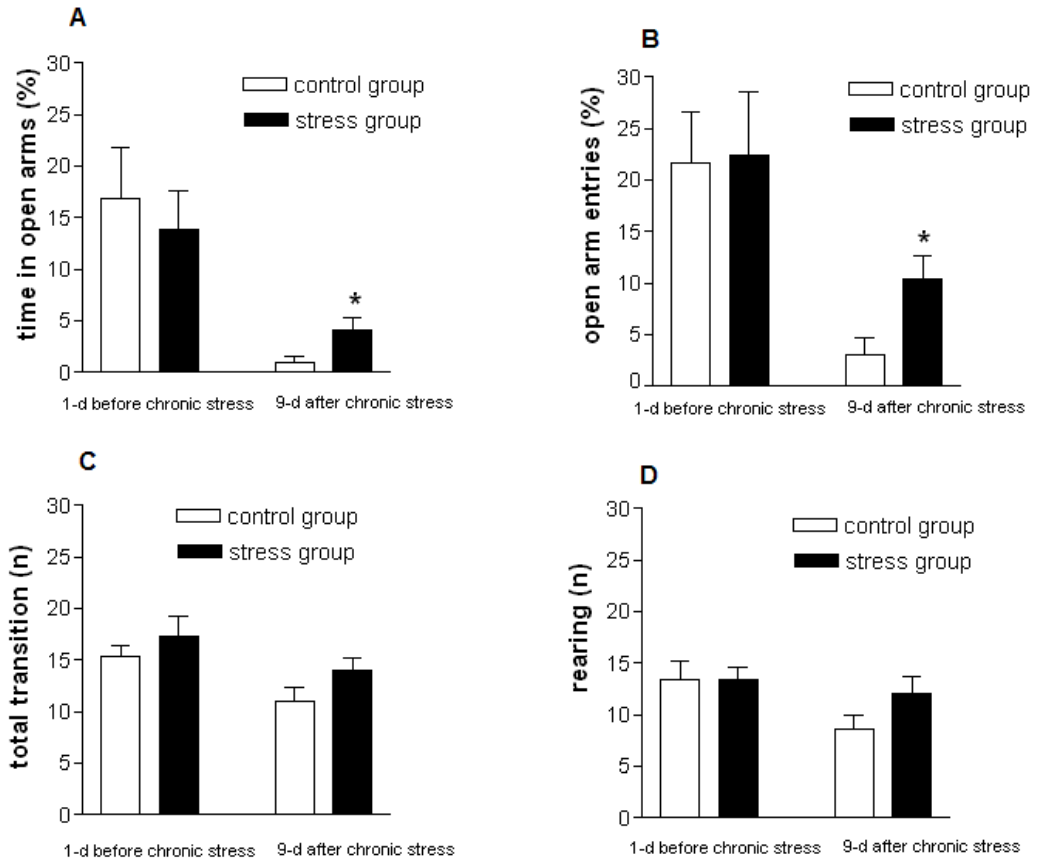

Figure 17. Elevated-plus maze test. Mice were tested in the elevated-plus maze 1 day before chronic unpredictable mild stress exposure and 9 days after chronic stress. **A** Percentage of time in the open arms (*p< 0, 05). **B** Percentage of open arm entries (*p< 0, 05). **C** Percentage of total transitions between closed and open arms. **D** Number of rearing bouts. 20 stressed mice and 13 control mice were tested.

4.2.3 Long-term effects of chronic unpredictable mild stress on depression-like behaviour in mice in the tail suspension test

As our study focused not solely on the short-term effects of chronic unpredictable mild stress we therefore analysed depression-like behaviour in the stress and control group 14 days after chronic unpredictable mild stress exposure. There were no differences in time spent in immobility in the stress and control group 14 days after termination of chronic stress tested with the Mann Whitney test (Fig. 18 A). A post-hoc analysis indicated no significant difference in time in immobility between the stress and control group (Fig. 18 B). There was also no difference in time spent in locomotion (moving while hanging) between control and stress group when having applied the Mann Whitney test (Fig. 18 C).

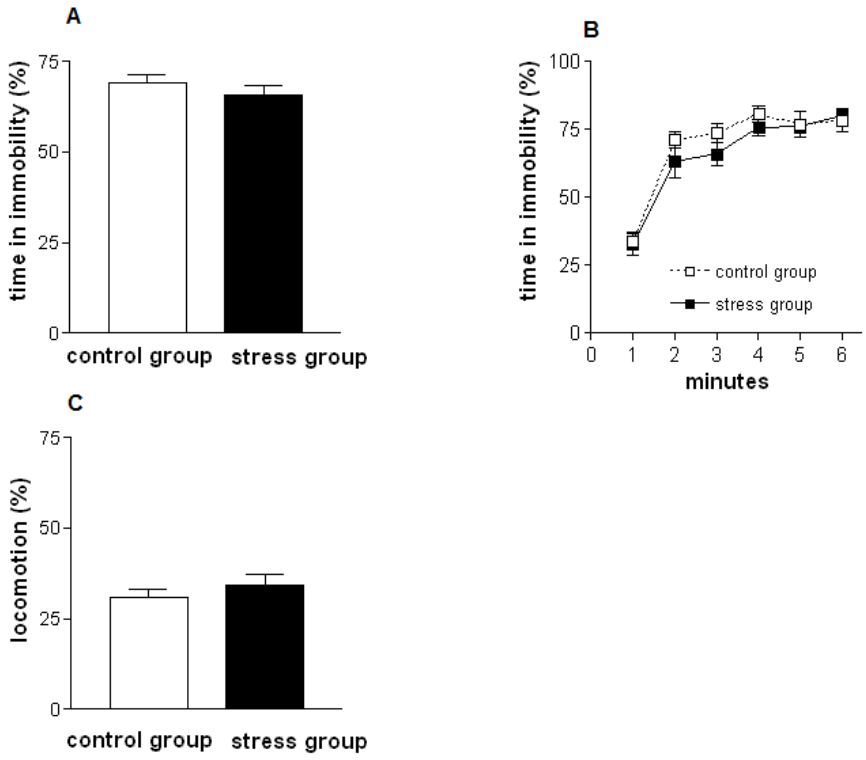

Figure 18. Depression-like behaviour in mice tested in the tail suspension test 14 days after termination of chronic unpredictable mild stress treatment. There were no significant differences in the percentage of time in immobility **A**+ **B** as well as in locomotion **C** in stress (n=20) and control (n=19) group when tested with the Mann Whitney.

4.2.4 Long-term effects of chronic unpredictable mild stress on body weight in C57BL/6J adult male mice

Chronic stress can induce body weight gain (obesity) and body weight loss (accompanied by anhedonia) which is considered to be linked with a desmetabolic state (Kyrou and Tsigos, 2008; Moreau, 1997).

4.2.4.1 Body weight during isolation

During isolation the control group (n=20) was kept in groups of 5 whereas the stress group (n=20) was isolated. The two-way ANOVA for repeated measures showed only a significant effect of days (p< 0, 001; Fig. 19 A) but not of treatment.

4.2.4.2 Body weight during chronic unpredictable mild stress

In the 13 days during the chronic unpredictable mild stress protocol the body weight of the stress and control group were measured on the 1st, 7th and 13th day of stress. Two way ANOVA for repeated measurements showed a significant effect in the stress group compared with the control group at t 13th day of chronic stress (p< 0, 0001; Fig. 19 B). The control group was still kept in groups of 5 mice during the 13 days of chronic unpredictable mild stress.

4.2.4.3 Body weight 14 days after termination of chronic unpredictable mild stress

In order to detect the long-term effects on the body weight of the chronic unpredictable mild stress mice of the stress and control group were not immediately killed after termination of chronic stress protocol but two weeks afterwards. The control group was immediately isolated after the end of the chronic unpredictable mild stress. Two-way ANOVA for

repeated measures showed a significant effect of group (p< 0, 0001) and an effect of the interaction between group and days (p< 0, 0001). Post-hoc analysis indicated that there is a significant effect of body weight in the stress group between the day of termination of chronic stress and 14 days after termination of chronic stress (p< 0, 001; Fig. 19 C).

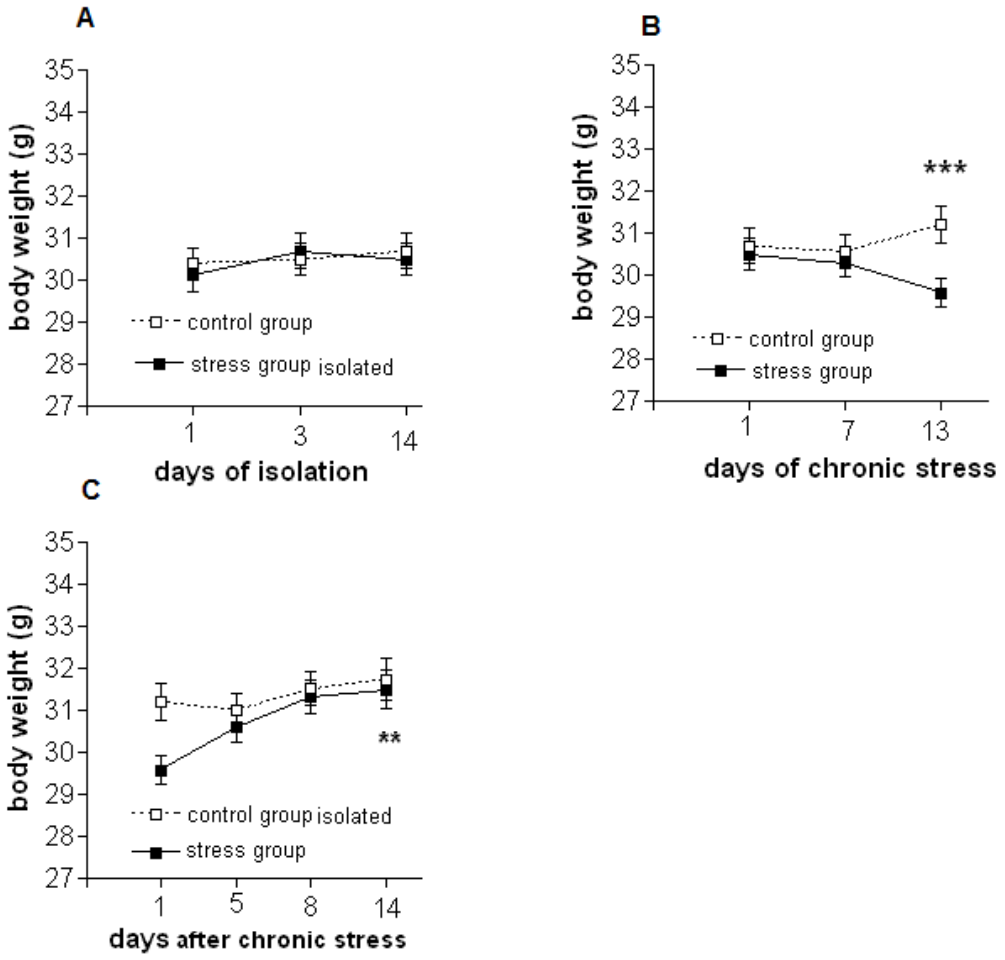

Figure 19. Body weight of control (n=19) and stress group (n=20) group were measured **A** during isolation **B** during chronic stress and **C** 14 days after termination of chronic unpredictable mild stress. **B** Two way ANOVA for repeated measurements indicated a significant effect of body weight between stress and control group on day 13 (***p< 0, 0001). **C** Post hoc analysis indicated a significant effect of body weight in the stress group from day of end of chronic stress until 14 days after termination of chronic stress (**p<0, 001).

4.2.5 Long-term effects of chronic unpredictable mild stress on weight of organs in C57BL/6J adult male mice

Preputial gland, adrenal glands, testes and spleen were dissected 14 days after termination of chronic stress to test the long-term effect of chronic unpredictable mild stress on weight of these organs.

4.2.5.1 Preputial gland

The study showed the weight of the preputial gland in the stress group was significantly reduced compared to the control group after 14 days of chronic stress ($p < 0,05$; Fig. 20 A).

4.2.5.2 Adrenal glands

Adrenal glands were removed after mice were killed after 14 days of the used chronic stress protocol. The weight of the adrenal glands in the stress group compared with the control group showed that stressed mice had significantly enlarged adrenal glands ($p < 0,001$; Fig. 20 B).

4.2.5.3 Testes

The testes of stress mice were not altered in comparison to the control group (Fig. 20 C).

4.2.5.4 Spleen

No differences were detected in the weight of the spleen of stress and control group (Fig. 20 D).

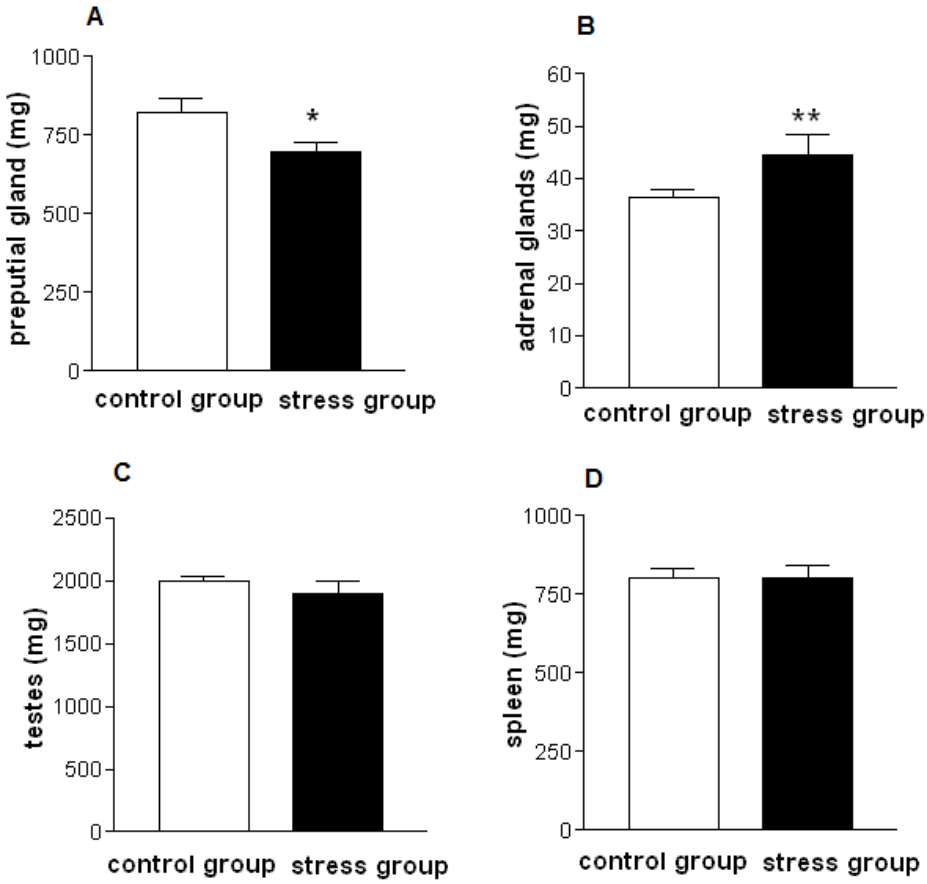

Figure 20. Organ weights of stress and control group after 14 days of termination of chronic unpredictable mild stress. Mann Whitney test showed a significant reduction in the weight of the preputial gland **A** (*p< 0, 05) whereas the adrenal glands **B** were significantly enlarged in the stress group (**p< 0, 001). Weight of testes **C** and spleen **D** did not differ between stress and control group on the long-term effect of chronic stress.

4.2.6 Long-term effects of chronic unpredictable mild stress on the plasma corticosterone concentration in C57BL/6J adult male mice

Changes in plasma corticosterone levels are used to indicate altered activity in the HPA-axis. Altered HPA-axis activity in turn mediates an impaired negative feedback regulation of corticosterone release. To evaluate a change in the release of corticosterone after chronic stress and after acute stress (here the tail suspension test) corticosterone in the plasma was measured 7, 30 and 60 min after the tail suspension test which was done 14 days of chronic unpredictable mild stress. The two-

way ANOVA indicated a tendency for a significant effect of the interaction between group and time (p= 0, 06; Fig. 21). Newman-Keuls post-hoc analyses indicated that levels of corticosterone are higher in the stress group compared with the control group 60 min after tail suspension (p< 0, 05; Fig. 21). Moreover, levels of corticosterone decrease from 30 to 60 min after tail suspension test in the control group (p< 0, 07; Fig. 21). The plasma corticosterone concentration did not differ between the time points in the stress group.

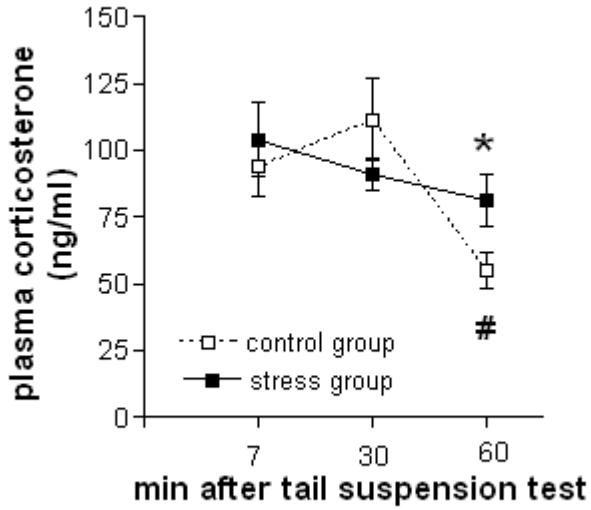

Figure 21. Plasma corticosterone concentration. To test the long-term effect of chronic unpredictable mild stress on the plasma corticosterone blood samples were collected 7, 30 and 60 min after the tail suspension test 14 days after termination of chronic stress. There was a significant decrease in the plasma corticosterone level 60 min after tail suspension in the stress group with Newman-Keuls post-hoc analyses (*p< 0, 05). Comparing the control group from 30 min to 60 min after tail suspension test a significant drop-off in plasma corticosterone level could be detected (# p< 0, 07).

4.2.7 Long-term effects of chronic unpredictable mild stress on mRNA expression of cell adhesion molecule NCAM and L1 corticosteroid receptors MR and GR in the hippocampus of C57BL/6J adult male mice

We had shown that the mRNA expression of cell adhesion molecules NCAM and MR were upregulated in the hippocampus 2 days after termination of chronic stress. To address the question whether gene expression of cell adhesion molecules and corticoids are shortly or permanent dysregulated by a 13 day period of chronic unpredictable mild stress we analysed the mRNA expression of NCAM, L1, MR (mineralocorticoid receptor) and GR (glucocorticoid receptor) in the right hippocampus 14 days after termination of chronic unpredictable mild stress. Mice were sacrificed 0, 30 and 60 min after the tail suspension test
There was a significant long-term reduction of NCAM mRNA expression in the hippocampus ($p < 0, 01$; Fig. 22 A) and unaltered L1 mRNA levels (Fig. 22 B). Mineralocorticoid receptor (MR) mRNA was significantly reduced in the hippocampus 14 days after chronic stress ($p < 0, 05$; Fig. 22 C) whereas mRNA expression of GR was not affected after 14 days of the end of chronic unpredictable mild stress (Fig. 22 D).

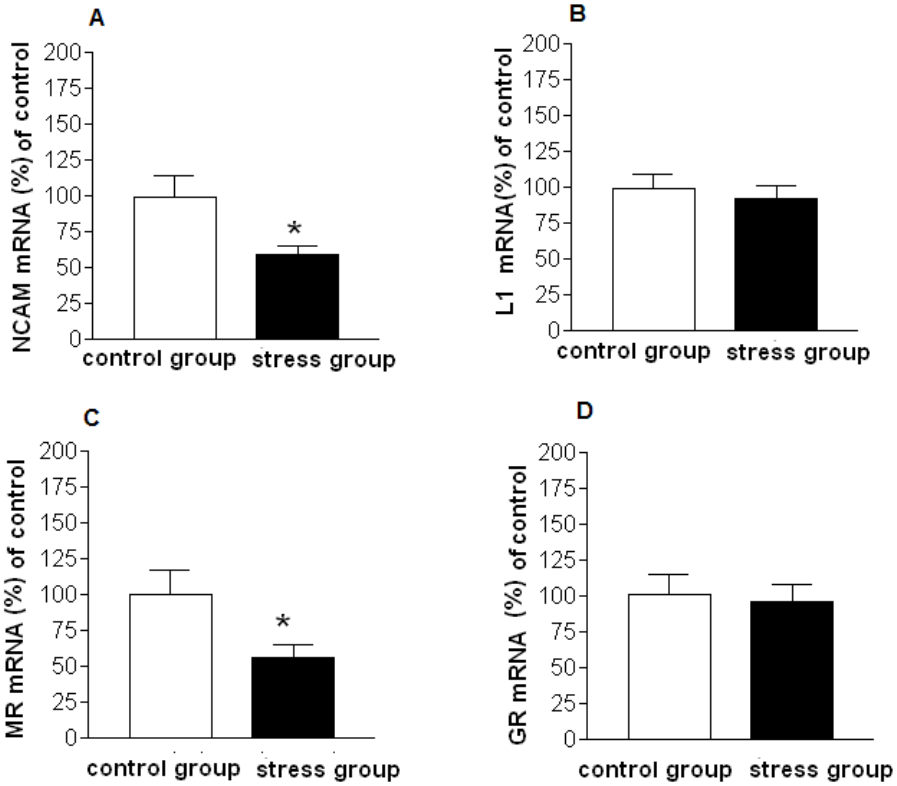

Figure 22. Hippocampal mRNA expression of NCAM, L1, MR and GR 14 days after termination of chronic unpredictable mild stress. The right hippocampi were removed to quantify mRNA levels for the NCAM, L1, MR and GR receptors. **A** Hippocampal mRNA expression of NCAM of the stress group was significantly decreased testing with Mann Whitney (*p<0, 01). **B** There were no differences in hippocampal mRNA expression of L1. **C** MR mRNA was significantly reduced in the stress group after 14 days of termination of chronic unpredictable mild stress (*p< 0, 05). **D** Expression of GR mRNA in the hippocampus was similar in stress and control group. (stress group: n= 11, control group: n=10).

V DISCUSSION

Chronic stress is a risk factor inducing structural, functional and molecular alterations of the brain which enhance the probability to develop psychiatric disorders like affective disorders (Maletic et al., 2007) or schizophrenia (Finlay and Zigmond, 1997). Taking into consideration that cell adhesion molecules play a key role in neuronal migration, neurite outgrowth promotion, neurite fasciculation, pathfinding, target recognition, synaptogenesis and myelination involved in neural development, synaptic plasticity and learning processes (Maness and Schachner 2007), it is most likely that the cell adhesion molecules NCAM and L1 may be involved in the dysfunctions of neuronal stress regulation and seem to play a potential pharmacological target for stress-related affective disorders. Previous studies have shown chronic stress-induced alterations on the expression levels of the neuronal cell adhesion molecules NCAM and L1 (Sandi, 2004; Sandi et al., 2001; Touyarot and Sandi, 2002 and Venero et al., 2002).

5.1 Chronic unpredictable mild stress induced depression-like behaviour in C57BL/6J adult male mice

In this study we aimed to establish a realistic animal model of non-traumatic chronic stress inducing depression-like symptoms in C57BL/6J mice. Thus we generated a new chronic unpredictable mild stress (CUMS) protocol based on the principles of chronic mild stress (CMS). In 1987, Willner and co-workers developed the chronic mild stress (CMS) protocol for rats, which includes a variety of low-grade stressors administered over a long period of time (Willner et al. 1987). An essential feature of the model is the exposition to different stressors which is a repeated presentation of a single stressor (Muscat and Willner 1992). In the present study, performed under the principles for scientific experiments on animals

issued by Hamburg ethical commission, we have modified the CMS procedure into chronic unpredictable mild stress (CUMS) by applying chronic low grade and timely unpredictable stressors over a 13-day-period for mice (manipulations with animals were done few minutes up to 24 hours per day). The established CUMS is based on the use of combination of different exogenous stress factors including environmental and social stressors in order to induce depression-like behaviour in mice. The environmental stressors (bright illumination, 24 hours light and cold water) of our CUMS paradigm differ in their intensity from other chronic protocols which consist of strong exogenous stressors like restraint foot shock and immobilization in a tube or water and food deprivation (Dunn and Swiergiel, 2008; Wood et al., 2008).

In our chronic unpredictable mild stress protocol we designed predatory and intrusive stressors (social stress, rat exposure, 24 hours exposure to rats, 15 hours exposure to rats and rat room) to induce depression-like behaviour in mice. Repeated social defeat in animals could be considered as a behavioural correlate of depressive symptoms in humans (Björkqvist 2001) expressed by feelings of worthlessness. The feeling of worthlessness is one symptom in the criteria of the diagnosis for major depression in the International Classification of Diseases (ICD-10) and Diagnostic and Statistical Manual of Mental Disorders (DSM IV).

In the forced swimming test which was used in our chronic unpredictable mild stress protocol as a stressor (cold water) and as a test for screening depression-like behaviour chronic stressed mice performed a depression-like behaviour at the last day of our chronic stress protocol in both projects. The fact that the forced swimming test was done not only on the first, seventh and last day of chronic unpredictable mild stress paradigm but also as a last stressor terminating chronic stress tempts to speculate that mice might be stressed by all stressors of our chronic stress protocol.

In order to distinguish between the influence of the forced swimming test and the chronic unpredictable mild stress protocol on

depression-like behaviour we also should have tested the control group in the forced swimming test. But in order to have a standard for comparison, the control group did not receive any experimental stressful stimuli.

To test the stress and control group additionally on depression-like behaviour the tail suspension test was performed after termination of chronic unpredictable mild stress in both projects. The tail suspension test was originally proposed by Steru et al., (1985) as a primary screening test of anti-depressant drugs and since it has become the most widely used model for assessing antidepressant-like activity in mice. The fact that the stress group showed a significant immobility as compared to the control group in the tail suspension test 2 days after termination suggests that our CUMS successfully induced depression-like behaviour on C57BL/6J mice.

5.2 Impairment of the hypothalamic-pituitary-adrenal axis after chronic unpredictable mild stress

Besides the effects of chronic unpredictable mild stress on the behaviour in mice we investigated several chronic stress-induced physiological parameters. In agreement with previous reports which have used chronic stress paradigms (Magariños and McEwen 1995a; 1995b; Sandi et al., 2001), we found a decrease in body weight, an increase in adrenal weight and altered plasma corticosterone levels. A possible explanation for the loss of body weight in stressed mice during chronic unpredictable mild stress exposure might be a stress-induced increase in energy consumption and stressed-induced anhedonia which is associated with the mesocorticolimbic dopamine reward system (Martin-Soelch, 2009). Anhedonia is characterising loss of interest or pleasure in daily activities which are symptoms often found in depressive patients. Even if anhedonia was not tested successfully in our project, a loss of body weight caused by decreased preference of food intake during chronic unpredictable mild stress exposure was previously shown by Moreau in 1997.

We measured a hypertrophy of the adrenal glands in chronic stressed mice 2 days and 14 days after chronic unpredictable mild stress procedure which was consistent with the increase concentration in plasma corticosterone after the tail suspension test. Corticosterone, secreted by the adrenal glands, is the end product of the HPA-axis and is strongly regulated by stress. The hypothalamic-pituitary-adrenal (HPA) axis, an important part of the neuroendocrine system, with the consequent release of CRH, ACTH and glucocorticoids plays an important role in the physiological stress response triggered by a wide range of psychosocial and physical stressors. The hypothalamus, pituitary gland and adrenal glands regulate stress-related physiological processes, including digestion, the immune system, mood and emotions, sexuality and energy resources. Moreover a HPA-axis dysfunction contributes to the neurobiology of psychiatric disorders, including major depressive disorder, post-traumatic stress disorder and schizophrenia (Paykel, 2003; Yehuda et al., 1991; Walker et. al., 2008). Plasma levels of ACTH and corticosterone well indicate the HPA-axis activity after stress. Plasma corticosterone responses to acute stress, such as after tail suspension test, and after chronic stress regime were used as an indicator of altered activity of the HPA- axis in studies by Rich and Romero (2005) and Retana-Márquez et al. (2003). In our study we observed an increased concentration of plasma corticosterone after 60 min in project 2 and a tendency to increase in 30 min after the tail suspension test in project 1. This observations support the idea that the chronic stress-induced impaired plasma corticosterone concentration might be a result of a deregulated negative feedback inhibition of the hypothalamic-pituitary-adrenal (HPA) axis.

The decrease in weight of preputial glands in C57BL/6J adult male mice of this study implies a stress-induced alteration in the synthesis of pheromones triggered by social defeat. Our finding, correlating with the study by Dijkstra et al. (1992) who observed reduced preputial glands but normal testes in subordinated male Wistar rats, affirms a submissive effect

through predatory and intrusive stressors of our chronic unpredictable mild stress protocol. Taking the weight reduction of the preputial gland and the depression-like behaviour with an altered HPA - axis 2 days after termination of chronic unpredictable mild stress together we clearly have shown physiological and behavioural alterations in the stressed mice linked to their exposure to our chronic stress paradigm.

5.3 Normal behaviour after 14 days of termination of chronic unpredictable mild stress

We were interested to analyse the long-term changes of our chronic unpredictable mild stress paradigm on C57BL/6J mice because up to now no studies have been conducted to investigate the long-term changes of chronic stress on C57BL/6J adult male mice. Therefore molecular, behavioural and physiological analyses were performed not only 2 days but also 14 days after termination of chronic unpredictable mild stress.

We made the interesting observation that previously stressed mice, which were left undisturbed for 14 days after the end of the CUMS exposure, showed no signs of significant depression-like behaviour in the tail suspension test two weeks after termination of chronic unpredictable mild stress. Furthermore the omission of chronic stress for 14 days may have resulted in normal behaviour (no „depression" in the previously stressed mice). This finding could indicate a recovery in the former chronically stressed mice. Further results which support the hypothesis of recovery from stress could be drawn from the normalization of the body weight and the increase exploratory behaviour of the stress group in the elevated plus-maze test (EPM) nine days after termination of chronic unpredictable mild stress. In view of the body weight increase 14 days post chronic stress, the question arises out of the contribution of the sugared water intake during five days to the normalization of the body weight in the stress group.

One possible explanation for the behavioural recovery in C57BL/6J mice might be, besides the sole effect of the absence of chronic unpredictable mild stress, their adaptation to the repeated stressful events causing a higher tolerance to stress (reduced stress-vulnerability). Active coping strategies in humans either behavioural or psychological strategies to challenge stressful events was described by Lazarus in 1966. Yet our results raise the question if these chronic stressed mice were able to develop rudimentary coping abilities.

Following from the observed normal behaviour of the previous chronic stressed mice, we could demonstrate that we generated a chronic stress paradigm which did not cause long-lasting behavioural and physical stress symptoms in mice. These findings in wild type mice bear resemblance to the capability of humans to adjust to stressful circumstances and point out that elimination of stress factors play an important role in the process of convalescing.

5.4 Long-term downregulation of mRNA expression of the neural cell adhesion molecule (NCAM) and mineralocorticoid receptor (MR) in the hippocampus of C57BL/6J adult male mice

In our second project we found 14 days after termination of exposure to 13 days of chronic unpredictable mild stress that the expression of NCAM mRNA in the hippocampus of C57BL/6J mice, as assessed by RT-PCR, was reduced. This observation confirms and extends previous findings, in which NCAM mRNA (Venero et al. 2002; Touyarot and Sandi 2002) is reduced by chronic stress procedure.

The hippocampal MR mRNA expression was increased 2 days after the termination of chronic unpredictable mild stress whereas MR mRNA was decreased after 14 days of chronic unpredictable mild stress. Depending on the type of the used stress paradigms, the duration of the stress exposure and the mouse strain MR mRNA was reported to be

elevated, not affected or downregulated (Bates et al., 2008; Herman et al., 1995; Paskitti et al. 2000). Moreover, we investigated no significant alterations in the mRNA expression on L1 and GR in the hippocampus in both experiments of our project although previous studies had shown an upregulation of L1 mRNA expression and downregulation of GR mRNA in the hippocampus after chronic stress treatment (Meyer et al., 2001; Sandi et al., 2001).

While NCAM stabilises synaptic contacts stress induces structural alterations in neurons including atrophy of apical dendrite in CA3 pyramidal cells and ultrastructural changes in mossy fiber terminals (Magarinos and McEwen, 1995b; McEwen and Magarinos, 1997). Therefore structural alterations in the hippocampus could be triggered by stress-induced reduction of hippocampal mRNA expression in NCAM. The fact that we measured no downregulation of hippocampal NCAM mRNA expression on the short-term effects on chronic stress could be due to the mild stressors used in our CUMS protocol. These socio-environmental stressors may not had lead to an increase of plasma corticosterone concentration up to the threshold level, which would be high enough to downregulate MR and NCAM mRNA expression in the hippocampus. A permanent increase of plasma corticosterone has been related to decrease NCAM and MR mRNA expression in the hippocampus (Hügin-Flores et al., 2004; Sandi et al., 2001). Chronic stress can further result in deterioration of neuronal plasticity and in a reversible or permanent neuronal loss via imbalance of MR and GR activity (Sousa et al., 2008). Although it was published that neurons expressing PSA-NCAM do not show the presence of MR protein (Garcia et al., 2004) this study cannot exclude the indirect action of MR downregulation on NCAM mRNA expression in neighboring neurons upon chronic stress. In this context, it would be interesting to investigate whether the long-term reduction of the expression of NCAM and MR genes after chronic stress exposure are responsible for the recovery of “normal” behavioral and physiological functions (in other words, responsible for the long-term homeostatic

response to the chronic stress), or whether they represent a residual “molecular trace” of the exposure to chronic stress and may be responsible for a relapse of pathological symptoms later in life or when the organism is exposed again to stressful stimuli.

In addition it needs to be discussed if our chronic unpredictable mild stress protocol stimulated a compensatory adaptive mechanism in mice towards the chronic environmental and social stressors by a short-term upregulation of the gene expressions for NCAM and MR in the hippocampus. Nonetheless the question if compensatory mechanisms due to chronic unpredictable mild stress exposure result in long-term downregulation of NCAM and MR needs to be further investigated.

We conclude that by using a combination of socio-environmental stressors we have designed a chronic unpredictable mild stress protocol that induced a profound behavioural and physiological stress response in C57BL/6J mice. At the same time our protocol was mild enough to allow behavioural and physiological recovery of wild type mice following the long-term termination of the chronic stress protocol. It might be interesting to study if our protocol might induce severe and persistent alterations in the brain of mice with genetic predispositions. Therefore our chronic unpredictable mild stress protocol might be a convenient tool to study the contribution of environmental and endogenous factors to the pathopsychology of stress-related psychiatric disorders based on the two hit hypothesis.

VI SUMMARY

Chronic stress induces depression-like behaviour in mice and simultaneously induces a pattern of changes in the expression of the cell adhesion molecules NCAM and L1 which correlates with the role of these molecules in the synaptic plasticity and neuroprotective mechanisms. In this context, dysregulations in NCAM and L1 gene expression are also implicated in stress-related psychiatric diseases like major depressive disorder, bipolar disorder and schizophrenia.

This study was designed to investigate the short- and long-term (2 and 14 days after termination of chronic unpredictable mild stress) effects of a new chronic unpredictable mild stress protocol (CUMS) generated to resemble non-traumatic stress conditions in humans, on mouse behaviour and the expression of the mRNA for the cell adhesion molecules NCAM and L1.

Analyses of behaviour (depression-like behaviour and anxiety), physiological parameters (body weight and weight of adrenal glands, preputial gland, testes and spleen), plasma corticosterone concentration as well as hippocampal mRNA expression of NCAM, L1 and the corticosteroid receptors MR and GR in C57BL/6J adult male mice were performed.

Analyses on short-term effects showed chronic stress-induced depression-like behaviour, a hypertrophy of adrenal glands, a decrease in the weights of preputial glands and body weight. Moreover, the plasma cortoicosterone release was dysregulated 2 and 14 days after chronic unpredictable mild stress. On the contrary chronic unpredictable mild stress-induced alterations on depression-like behaviour and body weight normalized 14 days after chronic unpredictable mild stress exposure.

At long-term we saw a reduction in mRNA expression levels of NCAM and mineralocorticoid receptor (MR) in the hippocampus whereas neither glucocorticoid receptors (GR) nor L1 mRNA expression were

changed at short- and long-term following chronic unpredictable mild stress protocol.

Summarising up, we created a chronic stress protocol which induced significant short-term and long-term behavioural, physiological and molecular responses in mice. Moreover we observed a recovery of behaviour and body weight in C57BL/6J adult male mice to unstressed levels following the long-term termination of the chronic unpredictable mild stress.

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IX APPENDIX

9.1 Abbreviations

ACTH	adrenocorticotropic hormone
am	ante meridiem
CAM	cell adhesion molecule
cDNA	complementary deoxyribonucleic acid
CHL1	close homologue of L1
cort	corticosterone
CRASH	Corpus callosum hypoplasia, mental Retardation, Adducted thumbs, Spastic parapelgia, Hydrocephalus
CRH	corticotropin-releasing hormone
CUMS	chronic unpredictable mild stress
CMS	chronic mild stress
d	days
DCC	deleted in colorectal cancer
DNA	deoxyribonucleic acid
DNAse	desoxyribonuclease
dNTP	desoxyribonucleotide triphosphate
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	Dithiothreitol
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
EPM	elevated-plus maze
FCOF	free choice open field
FGF-R	fibroblast growth factor receptor
Fig.	figure
FN III	fibronectin type III
GAS	general adaptation syndrome
GPI	glycophosphatidylinositol
GR	glucocorticoid receptor
HPA-axis	hypothalamic-pituitary-adrenal axis
HPRT	hypoxanthine guanine phosphoribosyl transferase

HRP	horseradish peroxidise
ICD	International Classification of Diseases
i.e. (id est)	that is
Ig	immunoglobulin
Ig(G)	immunoglobulin (G)
IL	interleukin
INF	interferon
kDa	kilo Dalton
LC/NE	locus ceruleus-norepinephrine
LTD	long-term depression
LTP	long-term potentiation
lx	Lux
MAG	myelin associated glycoprotein
min	minutes
MR	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
n	number
NCAM	neural cell adhesion molecule
NCAM-/- constitutive	NCAM knockout mice
NMDA receptor	N-Methyl-D-Aspartat receptor
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pm	post meridiem
PSA	polysialic acid
PVN	paraventricular nucleus
RIA	radioimmunoassay
RNA	ribonucleic acid
RNAse	ribonuclease
RPTP	receptor-like protein tyrosine phosphatase
RT	reverse transcription
RT-PCR	real-time reverse transcription polymerase chain reaction
s	seconds
SAS	sympathoadrenal systems

Sema3A	Semaphorin3A
TNF-alpha	tumor necrosis factor alpha
TGF-beta	transforming growth factor beta
Th1	T helper cell 1
TST	tail suspension test
UV	ultra violet
VASE	variable alternative spliced exon
°C	grad Celsius

9.2. Curriculum vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

9.3 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
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Ian Belle Stein