TITLE PAGE

Genetic alterations in the aetiology of depression

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Preface

This master's thesis was written by Anne Flou Kristensen during the 3rd and 4th semester of the Master of Science in Medicine with Industrial Specialization at the Department of Health Science and Technology, Aalborg University, Denmark. The experimental work was performed at the Laboratory of Neurobiology and the supervisor of the project was Associate Professor Jacek Lichota.
References are denoted according to the Harvard Referencing System.

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Abbreviations:

5-HT	5- hydroxytryptamine
AC	Adenylyl Cyclase
ACTH	Adrenocorticotropin Hormone
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors
AP-1	Activator Protein-1
Arc	Activity-Regulated Cytoskeleton associated protein
BDNF	Brain Derived Neurotrophic Factor
CaMK	Ca^{2+}/Calmodulin-Dependent Protein Kinases
cAMP	cyclic Adenosine Monophosphate
CMS	Chronic mild stress
CNS	Central Nervous System
CREB	cAMP Response Element-Binding protein
CRF	Corticotrophin-Releasing Factor
DG	Dentate Gyrus
DNMT	DNA Methyltransferases
EAAT	Excitatory Amino-Acid Transporters
ECS	electroconvulsive shock stimulation
EGF	Epidermal Growth Factor
GABA	γ-aminobutyric
GAD	Glutamic Acid Decarboxylase
Gal	Galanin
GC	Glucocorticoids
Glu	Glutamate
GR	Glucocorticoid Receptor
HAT	Histone Acetyltransferases
HDAC	Histone Deacetylases
HPA-axis	Hypothalamic-Pituitary-Adrenal axis
LTD	Long Term Depression
LTP	Long Term Potentiation
NGFI-A	Nerve Growth Factor-Induced protein A
NMDA	*N*-Methyl-*D*-aspartate receptors
NPY	Neuropeptid Y
PDGF	Platelet-Derived Growth Factor
PKA	Protein Kinase A
Rims	Regulating Synaptic Membrane Exocytosis
VEGF	Vascular Endothelial Growth Factor

Abstract

In Denmark approximately 3 % suffers from moderate to severe depression, and worldwide this number exceeds more than 100 million people. Very little is known about the pathophysiology of depression, and to date researches have not been able to identify a single gene or a single brain structure which can be the reason for depressive behaviour. Though it has been found that many brain structures and genes are affected by the disease and the complex pathophysiology of depression involves alterations of several different neural circuits. The symptomatology of depression thus goes beyond depressed mood and covers clinically significant weight gain/loss, insomnia or hypersomnia, fatigue or loss of energy, feelings of worthlessness or excessive guilt, and recurrent thoughts of death or suicide. During the resent years not much has happened in the treatment area and since the 1950'ties, where two types of antidepressants were discovered, no new strategies or novel treatments have been found. The tricyclic antidepressants and the monoamine oxidase inhibitors were coincidently found to be effective as anti-depressive agents. Though the side-effect profile of these antidepressants have improved, the present treatment still remains sub-optimal. Unfortunately, in order to discover any novel treatment strategies, a more comprehensive knowledge of the underlying genetic mechanisms resulting in depression is needed.

Through this study, the commonly used Chronic Mild Stress (CMS) animal model of depression and the novel animal model of Maternal Separation (MS) will be subjects of an expression analysis, where 13 genes will be investigated. These genes include four subunits of the AP-1 complex; c-Jun, c-Fos, FosB, and JunB, two downstream targets of the AP-1 complex; Galanin (Gal) and Neuropeptide Y (NPY). Furthermore, was the whole Glucocorticoid Receptor (GR) expression investigated and the fraction of GR containing exon 1_7 was also included. Two plasticity related genes; Activity-Regulated Cytoskeleton associated protein (Arc) and Regulating Synaptic Membrane Exocytosis-1α (Rims1α), and three other genes; cAMP Response Element-Binding protein (CREB), DNA Methyltransferase -3a (DNMT3a) and Glutamic Acid Decarboxylase-1 (GAD1) were also included in the assay. The expression of all genes were investigated in five different experimental animal groups; control group, MS anhedonic group, CMS anhedonic group, MS/CMS anhedonic group and MS/CMS resilient group. All genes are related to depressive behaviour through different functions of the hippocampus. Glucocorticoid Receptor exon 1_7 was further investigated for methylations at the promoter region, which was accomplished through sodium bisulfite treatment of DNA and thereafter sequencing of DNA. Lastly an optimization of a Native Chrommatin Immunoprecipitation protocol was initiated.

Interestingly, it was found that both the CMS and MS paradigm had an effect on expression of all genes. Especially the effect of the animal models on subunits c-Fos, FosB and JunB is interesting as the expression of these three genes were similar. Moreover, CMS and MS seemed to have distinct effects on the expression of some of the genes, indicating that though the animals shared an anhedonic profile, the underlying genetic alteration might be different. Moreover, the expression of all genes except c-Jun was higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group, which indicates that the genotypic response of the MS/CMS resilient animals differ from that of the MS/CMS anhedonic animals.

Table of content

1. Introduction

Depression is a severe mental disease affecting more than 100 million people worldwide and in Denmark approximately 3 % suffers from moderate to severe depression diagnosed of the basis of the International Classification of Diseases-10. (Bromet et al. 2011)(Olsen et al. 2007) The depressive episodes are most often recurrent, and followed not only by psychiatric co-morbidities but also somatic diseases. The symptoms of depression goes beyond depressed mood and covers clinically significant weight gain/loss or appetite disturbance, insomnia or hypersomnia, fatigue or loss of energy, feelings of worthlessness or excessive guilt, and recurrent thoughts of death or suicide. (Bromet et al. 2011) Due to the many devastating symptoms of depression the World Health Organisation has ranged depression as no. 5 of diseases with the highest Disability Adjusted Life Years-score. This score is defined as the sum of years of potential life lost due to premature mortality and the years of productive life lost due to disability. Furthermore, according to World Health Organisation depression will be no. 2 on that list at year 2020. (Olsen et al. 2007) Unfortunately, not much has happened in regards to finding an effective pharmaceutical treatment against depression. In the 1950'ies two novel antidepressants were discovered. The tricyclic antidepressants and the monoamine oxidase inhibitors were coincidently found to be effective anti-depressive agents. This discovery also gave the first clues into the complex chemical changes of depressive symptomatology and neural circuits affected by depression. Since then the side-effect profile of antidepressants has been improved, but the treatments still remain sub-optimal. (Nestler et al. 2002)
In the recent years, it has become more evident that early adverse events like neglect or decreased maternal care are correlated to cognitive impairments and social dysfunctions, which might appear later in life. Adverse events like maternal separation (MS) have been found to result in depression and anxiety, which might be anchored in altered gene expression and/or epigenetic modifications. (Zhang et al. 2004)(Marais et al. 2008)(Darnaudery and Maccari 2007) However, little is known about the genetic alterations underlying depression and possible epigenetic modifications associated with depression. Therefore, more comprehensive exploration of these mechanisms is essential to gain a better understanding of depression, which eventually will rear novel antidepressant agents.

1.2 The Heterogeneity of Depression

Depression is said to be a heterogenic disease, thus hampering our understanding of its aetiology and pathophysiology. The diversity of depressive behaviour represents a major barrier in the search for the optimal treatment. However, through neuroimaging, neuropathological, and lesion analyzes of the brain neural networks implicated in mood regulation and emotional behaviour have been found to be involved in the pathology of the depressed brain. (Dreverts and Furey, 2009) Though, the knowledge is still rudimentary as it is impossible to take a biopsy of the brain of patients with depression and if this was possible, there would be no consensus in the exact location of the pathology. Further, neuroimaging analyses tell us that more than one region if the brain is implicated in mediating the symptoms of depression. In parallel to the different regions of the brain implicated in depression, many different genes seem to be involved in depressive episodes. The search for specific genes related to depression is obscured by the complex aetiology of the disease. Despite the difficulties in determining related genetic abnormalities with certainty, epidemiologic studies have shown that depression in 40-50 % of the cases is genetic, making the disease very heritable. (Nestler et al. 2002) To obscure the pathophysiology of the

disorder even more, depressed patients do not necessarily share the same symptoms. Depression should be seen as a syndrome composed of multiple pathophysiologies with distinct causes. Thus attempts have been made to define different subtypes of depression based on certain sets of symptoms. Though, the subtypes are only based on the symptomatology of the patients and evidence is lacking in emphasizing that these subgroups actually reflect different states of the disease. (Nestler et al. 2002)

In the following sections the current knowledge about some of the different neural networks implicated in depression will be presented. Furthermore, cognitive alterations linked to depressive episodes will be described.

1.3 Neural Circuits Affected by Depression

As described above most symptoms of depression is related to abnormalities in mood. As the classic signs of depression include feelings of hopelessness, worthlessness, impairment of memory and learning, and suicidality most research has focused on changes in hippocampus and prefrontal cortex where these feelings arise. However, neuroimaging has revealed several other neuronal structures to be involved as well. These regions involve nucelus accumbens, amygdala, and hypothalamus, which are involved in reward, fear, and motivation. (Nestler et al. 2002) All implicated structures have been associated with volumetric reductions and studies have reported reductions of the hippocampus ranging in magnitude from 8-19 % in depressed patients. (Drevets and Furey, 2009) Furthermore, the metabolism of these areas is increased during depressed episodes and it has been found that the severity of the illness is positively correlated with an increased metabolic activity in neural structures associated with depression. The metabolic changes are reversed subsequent to a successful treatment with antidepressants. (Drevets and Furey, 2009)

Neurochemical circuits, which are altered in depressed patients, interact across the brain structures mentioned above. Figure 1 shows a simplified overview of the neural structures and the chemical circuits of depression. In the following sections the involvement of the serotonergic, glutamatergic and GABAergic, and glucocorticoid systems will be described in relation to depressive behaviour.

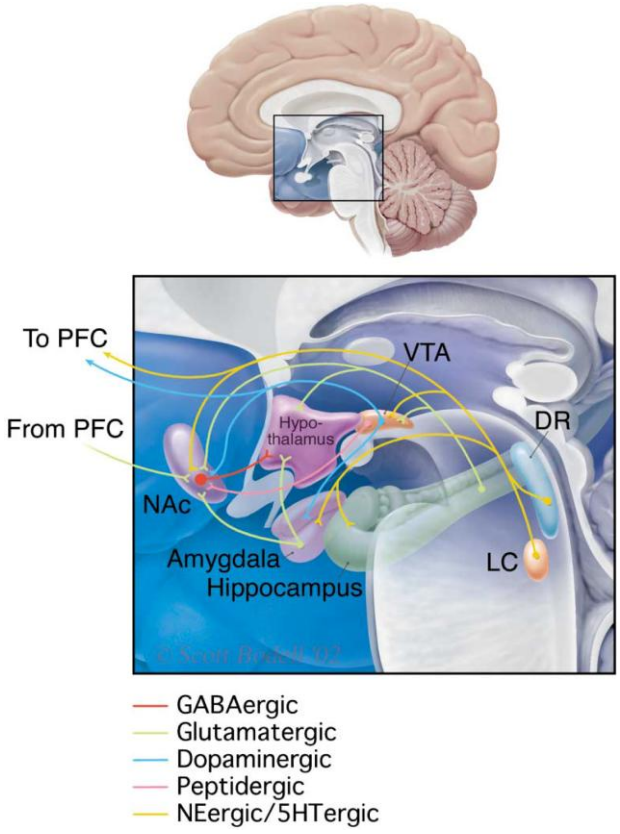


Figure 1 The figure only shows a subset of the many known interactions among the various brain structures and the neural circuits implicated in depression. The most commonly studied is the hippocampus and the Prefrontal Cortex (PFC). However, the Nucleus Accumbens (NAc), Ventral Tegmental Area (VTA), Amygdala, Dorsal Raphe (DR), and the Locus Coeruleus (LC) are also believed to be closely related to depressive behaviour. Some of the neural circuits implicated in depression, which interact across these structures, are listed below the figure. (Nestler et al. 2002)

1.3.1 The Serotonergic System

The monoaminergic serotonergic system affects most neural structures implicated in depression, see Figure 1. In patients suffering from depression the serotonergic activity is decreased and antidepressive monoamine oxidase inhibitors aim to increase the availability of 5-hydroxytryptamine (5-HT) at the synapse. (Nestler et al. 2002) The high complexity of the serotonergic system is accounted to the large number of receptors and their spliced variants, which activates many distinct signalling pathways. The 5-HT receptors are vastly all G-protein coupled receptors, which exerts their function through inhibition or activation of Adenylyl Cyclase (AC). (Raymond et al. 2001) Two of these receptors have been found to be associated with depressive behaviour, the 5-HT_{1A} and the 5-HT_7. (Raymond et al. 2001)(Weaver et al. 2007) The 5-HT_{1A} is believed to be involved in depression and in impairment of memory through alterations in the process of synaptic strengthening during Long-term potentation (LTP). (Raymond et al. 2001) In addition, the 5-HT_7 receptor, which is abundantly expressed in hippocampus, hypothalamus and in the neocortex up-regulates the expression of glucocorticoid receptor (GR) through demethylation of the nerve growth factor-induced protein A (NGFI-A) consensus sequence in the GR promoter region of neurons. Thus, making the serotonergic system an important contributor in the regulation of GR-dependent stress responses. The involvement of GR in depression will be described in section 1.3.3. (Weaver et al. 2007)(Zhang et al. 2010) The 5-HT_7 receptor has also been found to contribute to regulation of the circadian circuit, and as disruption of circadian rhythm is one of the most common symptoms of this emphasizes the importance the serotonergic system. (Masri and Sassone-Corsi, 2010)(Raymond et al. 2001) Another protein involved in the interactions of the serotonergic system is the neuropeptide Galanin (Gal). Ögren and co-workers found that Gal inhibits 5-HT release in the hippocampus. Moreover, they found that centrally administrated Gal has potent modulatory effects on 5-HT_{1A} mediated responses in the ventral hippocampus. (Ögren et al. 1998) Thereby, the serotonergic system plays an important role in modulating the depressive behaviour. However, details of why and how alterations of this system really contribute to depression remain to be elucidated. The mystery of the interplay between depression and the serotonergic system is only accentuated by the fact that the monoamine oxidase inhibitors were found coincidentally.

1.3.2 The Glutamatergic and GABAergic System

Glutamate (Glu) is a major excitatory neurotransmitter in the central nervous system (CNS), whilst γ-Aminobutyric (GABA) is an inhibitory neurotransmitter. The glutamatergic and the GABAergic systems are in close collaboration with each other. The glutamatergic system is comprised of both metabotropic and ionotropic receptors, where the ionotropic receptors can be subcategorised into α-Amino-3-hydroxy-5-Methyl-4-isoxazolepropionic Acid receptors (AMPA), *N*-Methyl-*D*-Aspartate receptors (NMDA), and Kainate receptors. (Sanacora et al. 2008) Meanwhile, the GABAergic system is also comprised of ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. The GABAergic system is comprised of interneurons, functioning as a regulatory "on/off" switch of e.g. the glutamatergic system. GABAergic transmission has been found to be decreased in depression, especially deficits in $GABA_A$ receptor signalling and GABA levels in the hippocampus. This reduction in GABA levels correlated with an increase in Glu levels, as Glu serves as a precursor for GABA synthesis at GABAergic neurons. Hence, reduced GABA synthesis decreases its inhibitory function and the conversion of Glu into GABA, leaving the glutamatergic transmission amplified in depressed patients, and the GABAergic system abnormally reduced. (Femenia et al. 2012)(Möhler 2011)

Both systems are closely implicated in emotional and cognitive behaviour. Under normal conditions Glu is associated with e.g. neuroplasticity, memory formation, and learning. However, the pathologic elevation of

Glu in depression is known to trigger rapid or delayed neurotoxicity. (Sanacora et al. 2008) In the normal glutamatergic system glial cells are responsible for clearage of Glu from the extracellular space via the Glu reuptake Excitatory Amino-Acid Transporters (EAAT). The quantity of these transporters was found to be decreased in the cerebral cortex of depressed patients, resulting in excitotoxicity. (Femenia et al. 2012) Additionally, the elevated metabolism seen in the depression-related neural structures is caused by this elevated Glu neurotransmission. (Drevets and Furey, 2009) The mechanisms behind LTP induced consolidation of learning and memory have been proposed to evolve around the interactions of glutamatergic receptors NMDA and AMPA (Johansen et al. 2011). Moreover, GABAergic neurotransmission has been proven to play an important role in LTP. Researchers have found that reduction of ionotropic α_5 $GABA_A$ receptor in the hippocampal regions CA1/CA3, see Figure 3B, lead to enhanced spatial memory and enhanced fear conditioning in mice. Thus implying that GABAergic inhibition is a key determinant of cognitive behaviour. (Möhler et al. 2011)

An important contributor to maintenance of the Glu/GABAergic homeostasis is the Glutamic Acid Decarboxylase (GAD) enzymes. GAD is the rate limiting enzyme in the synthesis of GABA from Glu. Interestingly, it has been found that GAD1, which is abundantly expressed in the hippocampal structure, is regulated with maternal care. In a study by Zhang et al. 2010 they found that decreased maternal care resulted in a decrease in GAD1 mRNA in the hippocampus. The decrease in GAD1 expression was caused by increased methylation of the GAD1 promoter region. Moreover, they found that increased maternal care was associated with increased 5-HT and NGFI-A expression in the hippocampus, and as the promoter region of GAD1 contains a NGFI-A consensus sequence, they speculated that maternal care influences the GABAergic system by altering GAD1 promoter methylation through maternally induced activation of NGFI-A. (Zhang et al. 2010)

1.3.3 The Glucocorticoid System

The glucocorticoid system regulates and supports various functions essential to existence including cardiovascular, metabolic, and homeostatic functions. In regards to depression, the most important function of the glucocorticoids (GC) is to regulate the neuroendocrine Hypothalamic-Pituitary-Adrenal axis (HPA-axis), see Figure 2. The HPA-axis mediates the stress response and regulates

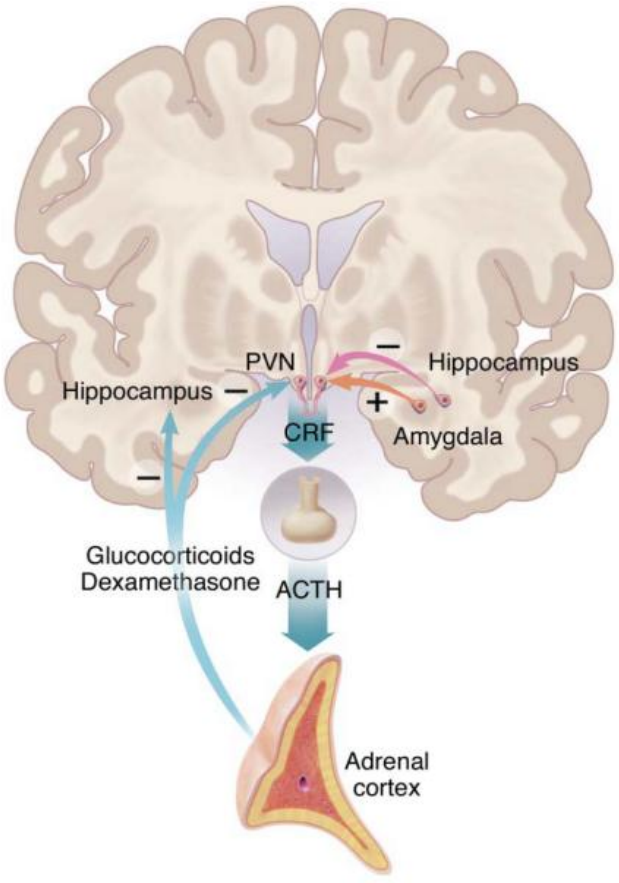


Figure 2 The regulation of the Hypothalamic-Pituitary-Adrenal axis (HPA-axis) has been shown to be changed in depression. Stress responses initiates in the Paraventricular Nucleus of the hypothalamus (PVN) with release of Corticotrophin-Releasing Factor (CRF), which mediates the secretion of Adrenocorticotropin Hormone (ACTH) from the pituitary gland. ACTH effects the release of Glucocorticoids from the adrenal cortex, thus inhibiting CRF secretion. Dexamethasone is a synthetic Glucocorticoid, which also exerts negative feedback in CRF release (Nestler et al. 2002)

mood and emotions. A stimuli percepted as a stressful event releases Corticotrophin-Releasing Factor (CRF) from the paraventricular nucleus of the hypothalamus. CRF reaches the anterior pituitary gland via capillary networks and stimulates the release of Adrenocorticotropin Hormone (ACTH) into the circulation. The adrenal gland releases glucocorticoids (GC) in response to the circulating ACTH. A negative feedback mechanism mediated by the GCs onto higher brain centres like hypothalamus and hippocampus ensures the equilibrium of the system. Thus, the GC level seen under normal physiological circumstances enhances hippocampal inhibition of HPA activity, which in turn down-regulates the stress response. (Femenia et al. 2012) However, in depressed patients the HPA-axis fails to succumb this task consequently resulting in an increased GC level, which hyperactivates the HPA-axis. Alternately, a hyperactive HPA response contributes to depressive behaviour. (Weaver, 2009)

Dysregulation of the HPA-axis

An altered GC system leaves the HPA-axis dysregulated and thereby hypersensitive towards stress. Furthermore, prolonged stress generates additional production of GC, resulting in a vicious circle of an even more damaging stress response. The sustained elevated GC levels seen in depressed patients, as a consequence of continuous exposure to stressful events, induces damage to the neurons of hippocampus, especially pyramidal neurons of the CA3 region, see Figure 3B. This neural damage is thought to involve reduction of dendritic branching followed by loss of essential glutamatergic synaptic inputs, which is important for cognitive behaviour. (Nestler et al. 2002)(Drevets and Furey, 2009) The lack of inhibitory HPA regulation from GC also elevates cortisol levels, which also results in devastating damages. An elevated cortisol level over sustained periods of time might be toxic for hippocampal neurons. Based on normal functions subserved by the hippocampus, damaged hippocampal neurotransmission is suspected to contribute to some of the cognitive behavioural changes of depression. Even further, scientists have speculated that the neurotoxic effect of elevated GC and cortisol levels might lead to the volumetric decreases of hippocampus. (Nestler et al. 2002)

It has been found that in some cases of depression administration of the potent synthetic GC dexamethasone is unable to suppress plasma levels of cortisol and ACTH, see Figure 2, which might indicate that the glucocorticoid receptor (GR) is altered. (Nestler et al. 2002)(Drevets and Furey, 2009) The expression of GR is determined by 11 distinct first exons, most of these exons are located in a 3 kb CpG island upstream of exon 2, which exhibits substantial promoter activity, see Figure 3A. The promoter regions of these exons regulate the expression of GR in a tissue-specific manner. Exon 1_7 has been shown to be expressed at significant levels in hippocampus, but was expressed at either low or undetectable levels in other tissues. Furthermore, it has been proven that hippocampal exon 1_7 is the only of the alternative first exons of GR that is affected by perinatal handling of rat pups. (Weaver et al. 2001) In a study by Weaver et al. it was proven that early adverse events like poor maternal care led to decreased GR density in hippocampus, permanently altering the HPA-response to stress in a depression-like manner. They found that pups deprived of licking and grooming from their mother suffered from increased methylation and decreased acetylation of GR exon 1_7, thus resulting in the attenuation of GR expression. (Weaver et al. 2004) DNA hypermethylation and histone hypoacetylation are two epigenetic modifications leading to blocked gene expression, which will be described in section 1.5. The decreased GR expression, by means of increased GR exon 1_7 methylation, might be attributed to methylation of the NGFI-A motif embedded in the exon 1_7 promoter. (Weaver et al. 2004)(Zhang et al. 2010) Though it is important to emphasize that results obtained in our laboratory indicates that the NGFI-A motif is not methylated under the influence of stress.

(Henningsen et al. 2012) It has been speculated that 5-HT is partially associated with the methylation of GR exon 1_7, as depletion of 5-HT reduces the density of GR in hippocampus in a cyclic Adenosine Monophosphate (cAMP) dependent manner (Weaver et al. 2001). The GR expression might also be regulated by various other transcription factors. The GR gene sequence carries different response elements of many cAMP related transcription factors e.g. cAMP Response Element-Binding protein (CREB), whereby cAMP might increase GR expression. (Weaver et al. 2001)

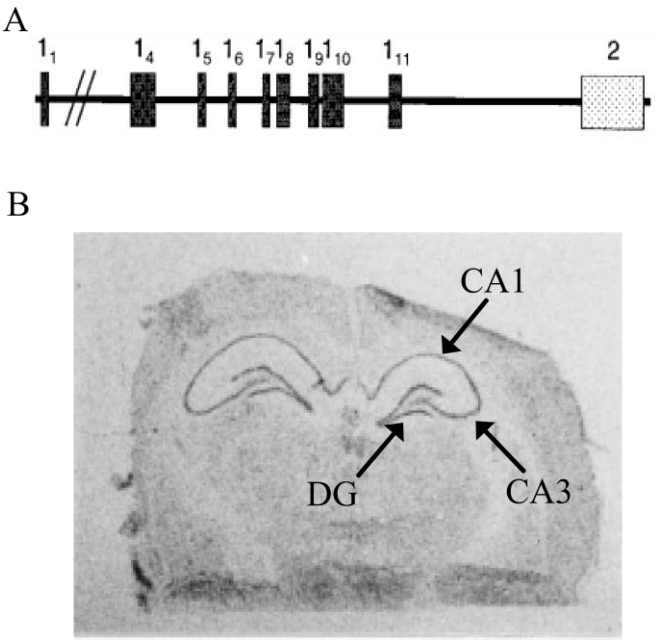


Figure 3 The figure shows A) The distrubution of first exons of Glucocorticoid Receptor (GR) up-stream of exon 2. Exon $1_{1\text{-}11}$ is located in a CpG island. B) *In situ* hybridization of the distribution of GR exon 1_7 mRNA in the hippocampus, The arrows marks position of the CA1, CA3 and the Dentate Gyrus (DG). Modified from McCormick et al. 2000.

The Neuropeptide Y (NPY) has also been suggested to contribute to the regulation of the HPA-axis. However, NPY is thought to exhibit stress reducing functions via positive regulation of the HPA-axis. NPY is a widely distributed neuropeptide in the CNS and administration of NPY has been related to increased food intake, modulation of cognition, inhibition of neuronal excitability, and regulation of HPA-axis by increasing the release of cortisol. (Cohen et al. 2012) NPY exerts its functions through five different NPY receptors, which display distinct functions. NPY receptor Y_1 has been found to be increased with administration of antidepressants while NPY receptor Y_2 is decreased. It has been found that hippocampal NPY levels are decreased in depression, and that this decrement can be restored with chronic electroconvulsive shock stimulation (ECS), which has been proven to be an effective therapy against depression. (Redrobe et al. 2002) Moreover, it has recently been suggested that the NPY genotype may by associated to antidepressant medication response. This serves as potential NPY-based biological markers, which perhaps can contribute in discovery of novel antidepressant agents for the patients who do not respond to current treatment available. (Mickey et al. 2011)

Not only neural circuits are altered in depression, specific functions of the different structures influenced by depressive behaviour are also inclined. The hippocampal structure and its specific functions are especially under the influence of depression. Many of the functions of hippocampus relay on neuroplasticity. In the following sections the role of neuroplasticity in depressive behaviour will be described.

1.4 Impaired Neuroplasticity

Neural plasticity is a critical process for adaptation, memory formation and learning. It covers both functional connectivity of synapses in terms of synaptic plasticity and short and long term morphological changes, all of which are altered in depression. A decrease in synapse density has been observed in depressed patients, which increases the individual vulnerability towards negative input. Additionally, researchers have found that in a genetic rat model of depression, LTP was attenuated in the CA1 region of

hippocampus. In the same hippocampal region long term depression (LTD) was enhanced after exposure to chronic mild stress (CMS) in rats. Hence, facilitation of LTD over LTP in hippocampus as well as decreased synaptic density might cause severe cognitive deficits. (Femenia et al. 2012) Some of the more important regulators of plasticity are transcription factor CREB and its down-stream target neurotrophin Brain Derived Neurotrophic Factor (BDNF). CREB and BDNF are important regulators of consolidation of learning and memory, see section 1.4.1. (Nestler et al. 2002) In addition to these, Regulating Synaptic Membrane Exocytosis-1α (Rims1α) of the Rims gene family has been found to play an important part in regulating synaptic plasticity. The Rims gene family consists of four Rims genes, which are involved in regulating presynaptic Ca^{2+}-channel density, thereby regulating neurotransmitter release. The Rims1 gene encodes two isoforms, which mainly exerts same cellular functions. Both Rims1α and Rims1β are involved in short term plasticity and synaptic strengthening. However, Rims1α carries a N-terminal α-helix domain, which binds to synaptic vesicle protein Rab3 and this interaction is essential for presynaptic long-term plasticity. (Kaeser et al. 2008) The binding of Rims1α to Rab3 mediating presynaptic long-term plasticity has been linked to the CA3 region of hippocampus, whilst in the CA1 region the same interaction mediates short term synaptic plasticity and neurotransmitter release. (Kaeser and Südhof, 2005) Processes like short and long term potentation, synaptic plasticity, and consolidation are essential for maintenance of cognitive behaviour, and therefore understanding the underlying processes is fundamental in order to understand depression. Patients suffering from depression often battle memory and learning impairments due to alterations of these processes, two functions evidently linked to functions of hippocampus.

1.4.1 Memory and Learning

Depressed patients have as earlier mentioned decreased ability to form new memory and learn. The processes of memory formation and learning are mainly thought to be established through NMDA-dependent LTP, and one of the main contributors to these cellular processes is the transcription factor CREB. Kinases like Protein Kinase A (PKA) and Ca^{2+}/Calmodulin-Dependent Protein Kinases (CaMK) induce phosphorylation of CREB, which triggers binding of CREB to CREB binding protein (CBP) resulting in transcription of synapse remodelling and stabilization related genes, terminating in consolidation. (Johansen et al. 2011) Thus phosphorylation of CREB enhances formation of synaptic plasticity in the hippocampus, which alleviates the ability to learn and to form new memory. Interestingly, the administration of CREB in hippocampus and Dentate Gyrus (DG) has been shown to induce antidepressive effects. One down-stream target of CREB is BDNF, which is involved in neuronal survival, and remodelling of synapses. BDNF is like CREB a major contributor to LTP in hippocampus, and decreased BDNF levels result in learning deficits. Thus, learning and memory impairments induced in hippocampus in depression are mediated through phosphorylation of CREB, which leads to decreased BDNF expression alternately resulting in attenuation of LTP. (Nestler et al. 2002) The increased vulnerability to stress subsequent to impaired HPA response leaves the cortisol level increased, which have been found to decreases BDNF expression even further in the hippocampus of depressed patients. (Holderbach et al. 2006) CREB and BDNF are therefore major contributors to alterations of cognitive behaviour during depression. Moreover, Kinney and co-workers found that spatial learning and memory formation, which are essential functions of the ventral hippocampus, are impaired by Gal in a AC-dependent manner. They speculated that this impairment was caused by phosphorylation of CREB. (Kinney et al. 2009)(Fanselow and Dong, 2010) Thus, phosphorylation of transcription factor CREB is involved in enhancement of some signalling cascades while reducing others.

Stabilization of BDNF-induced LTP has been shown to depend on early synthesis of Activity-Regulated Cytoskeleton associated protein (Arc) in DG. Additionally, late phase synthesis of Arc is associated with consolidation of LTP, which is also induced by BDNF. Arc is categorized as an immediate-early gene, and is presumably involved in both LTP and LTD, making Arc a dynamic regulator of synaptic homeostasis. (Coppens et al. 2011)(Bramham et al. 2008) Translation of Arc is thought to occur at active synapses, as Arc mRNA seems to accumulate at activated neurons along the dendritic arbor. This is thought to be the crucial mechanisms behind Arc-dependent long term modifications specific to synapses. (Dyrvig et al. 2012) In addition, Arc is also associated with AMPA glutamate receptor trafficking. Expression of Arc cause increased surface AMPA endocytosis thereby reducing the level of surface AMPA, thus dampening pathological overactivation of the AMPA-related glutamatergic systems. (Bramham et al. 2008) Lastly, Arc also seems to be a downstream target of the transcription factor CREB. (Lam et al. 2009)

Another transcription factor known to be implicated in memory and learning is the Activator Protein-1 (AP-1). AP-1 is either a hetero- or homodimeric protein complex comprised primarily of a Fos and a Jun or of two Jun proteins. Members of the Fos family is: c-Fos, FosB, Fra-1, and Fra-2, while the Jun family consists of c-Jun, JunB, and JunD. (Raivich and Behrens, 2006) Expression analyses of genes of the Jun and Fos families have indicated that they are not involved in the induction of LTP because their expression have only weak or no correlation with the magnitude of the LTP. Instead, the proteins are speculated to regulate the duration and stabilization of LTP. (Herdegen and Leah, 1998) Of all AP-1 proteins c-Jun is the most abundantly expressed and it is involved in neuronal differentiation and survival, and in relation to depression c-Jun is involved in LTP and memory formation. c-Fos is also involved in memory and learning processes. It has been shown that mice produce a 4- to 5-fold increase in c-Fos and c-Jun expression subsequent to a behavioural bar pressing task. This increase in c-Fos and c-Jun expression was only observed in hippocampus, but not in other areas of the CNS. The expression occurred in the CA3 and CA1 regions, and was very high in the DG. Additionally, injection of a neurotoxin, which enhances post-training learning, called apamin has been shown to increase the expression of c-Fos and c-Jun in trained mice compared to untrained controls. Additionally, c-Jun:c-Fos dimers carries a c-fos and a CRE element, thus, as many other genes altered in depression, some dimers of the AP-1 complex are under the influence of transcription factor CREB. (Herdegen and Leah, 1998)

1.4.2 Neurogenesis

Neural plasticity covers not only small-scale changes like synaptic changes, but also changes in generation of adult born neurons. The process of neurogenesis is thought only to occur in the DG of hippocampus, olfactory bulb, and in the subventricular zone. Exposure to stressful events have been seen to decrease neurogenesis and further is neurogenesis of the DG thought to contribute to the volumetric changes of hippocampus seen in depressed patients. In vivo models of stress induction propose that exposure to CMS paradigm suppresses neurogenesis in DG, and that antidepressants restores these changes. (Holderbach et al 200)(Bergström et al. 2007) Generation of new adult neurons are thought to contribute to cognitive behaviour, and it has been found that reduction or blockade of neurogenesis affects fear conditioning, synaptic plasticity in DG and spatial long term memory. On the contrary induction of neurogenesis enhances survival of adult born neurons in DG. (Femenia et al. 2012) Neurotrophins, like BDNF, are thought to play an important role in mediating neurogenesis, as altered BDNF expression is associated with impaired neurogenesis. However, neurotrophins do not solitarily regulate neurogenesis, the angiogenic factor Vascular Endothelial Growth Factor (VEGF) is also essential for neurogenesis. The amount of VEGF

has furthermore been shown to decrease in response to stress. (Kunugi et al. 2010) In a study by Snyder et al. they found that in a neurogenesis-deficient mouse model the HPA response was altered. Mice lacking the ability to generate new adult neurons had an increased corticosterone, which is the is the dominant glucocorticoid in rodents, level after exposure to a stressor compared to wild-type controls, indicating that neurogenesis in DG might play a role in regulating the HPA-axis stress response. (Snyder et al. 2011) Thus, emphasizing the significance of neurogenesis in depression.

The mechanisms by which depression alters memory, learning and plasticity are still unclear. However, it remains certain that these functions are impaired in depression; Figure 4 summarizes some of the pathways, which are thought to be affected by depression. Whether the molecular changes are caused by impairments of co-factors or by epigenetic changes remains to be elucidated.

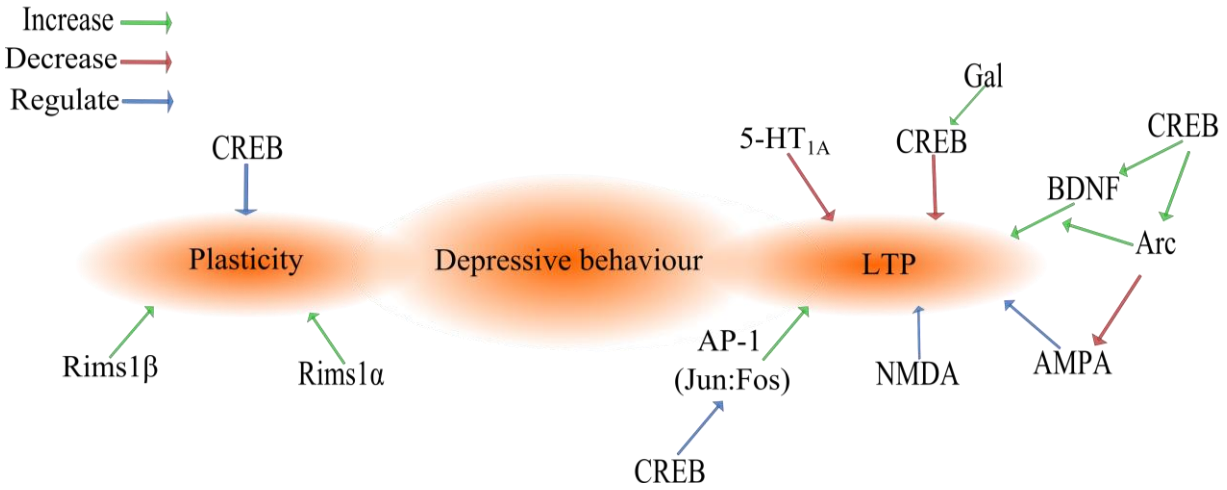


Figure 4 The figure summarizes regulatory paths of plasticity and LTP, which could be involved in impairments of cognitive behaviour leading to depression. Abbreviations: CREB: cAMP Response Element-Binding protein. Rims1: Regulating Synaptic Membrane Exocytosis1. 5-HT: 5- hydroxytryptamine. Gal: Galanin. BDNF: Brain Derived Neurotrophic Factor. Arc: Activity-Regulated Cytoskeleton associated protein. AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. NMDA: *N*-Methyl-*D*-aspartate receptors. AP-1: Activator Protein-1. The figure is made on the basis of 1.4, 1.4.1, and 1.4.2.

1.5 Epigenetic Regulations of Gene Expression

Epigenetic changes cover both stable and dynamic chromatin modifications independent of the underlying DNA sequence. The epigenetic modifications controlling gene expression is often referred to as the epigenome. Chemical changes of the epigenome involve changes in the chromatin structure, thus leaving the underlying DNA sequence intact. The chromatin structure is basically involved in packaging of DNA into a smaller unit to fit into the nucleus, prevention of damage to the DNA, strengthening of DNA, and to control replication and gene expression. (Riccio, 2010) Chromatin is comprised of nucleosomes, which are the core units. Nucleosomes consists of ≈ 150 bp of DNA wrapped ≈ 1.75 times around a histone octamer, which is the nucleosomal core. So far five classes of canonical histones have been identified: histone-1 (H1), histone-2A (H2A) and -2B (H2B), histone-3 (H3), and histone-4 (H4), and each histone octamer contains two of H2A, two H2B, two H3, and two H4. Modifications of the epigenome conduct gene accessibility mainly through DNA methylations and/or histone modifications, resulting in a dynamic chromatin structure. The activity of the dynamic chromatin leaves the DNA either accessible to transcription or condensed,

euchromatin or heterochromatin, respectively. (Weaver, 2009) The mechanisms behind DNA methylation and histone modifications will be elaborated in the following section.

1.5.1 Regulation of Transcription through DNA Methylation

DNA methylations are covalent bindings of a methyl group to the carbon 5 position of cytosine residues of 5'-cytosine-phosphodiester-guanine-3' (CpG), see Figure 5, and in humans 60-80 % of the cytosines of palindromic CpG sites are methylated. (Weaver, 2009) In general, it appears that hypomethylation of a gene resolves in an active gene, whereas hypermethylation relates to silencing of the gene. CpG sites both appear in so called CpG islands and as single sites along the DNA sequence. The CpG islands are ~ 1 kb in length and appear in 5' ends of genes, closely related to or in 40 % of gene promoters. (Weaver, 2009)(Lubin et al. 2008) The addition of a methyl group to the 5' positioned cytosine is initiated by *De Novo* DNA Methyltransferases (DNMT), see Figure 5. Hereafter, methylation of the complementary strand can commence, mediated by maintenance DNMT's. When both strands are methylated the modification is extremely stable and requires a high degree of energy to be reversed and demethylated. (Day and Sweatt, 2010) Initially, it was thought that methylations were irreversible and stable changes of the DNA structure. However, it has been found that the methylations can be reversed by either reducing the activity of DNMT or removal of nuclear proteins like the transcriptional repressor methyl-CpG binding protein 2 (MeCP2) from methylated CpG sites. Thus, contributing to the dynamicity of the epigenome. (Riccio, 2010) In mammalian cells three types of DNMT's, which methylate DNA, exist: DNMT1, DNMT3a, and DNMT3b. DNMT3a and DNMT3b is believed to be involved in *de novo* methylations while DNMT1 is involved in maintenance of methylations i.e. methylation of the complementary strand. (Weaver, 2009) Interestingly, the genomic sequence of the DNMT3a gene has been found to contain two isoforms, DNMT3a1 and DNMT3a2. Oliveira and co-workers 2012 found both isoforms to be significantly reduced in aged mice, and that the isoform DNMT3a2 was involved in cognitive impairments related to aging. They further tested the involvement of DNMT3a2 in hippocampal memory of young mice, and found that a decrease in DNMT3a2 resulted in impaired long term memory formation. They also found that the reduction in DNMT3a2 resolved in a decrease in the plasticity related genes BDNF and Arc, linking DNMT3a2 to accessibility of these genes. This indicates that the methylational status of a gene may be more complex than previously thought, as methylations possibly also can be associated with increased transcriptional activation.

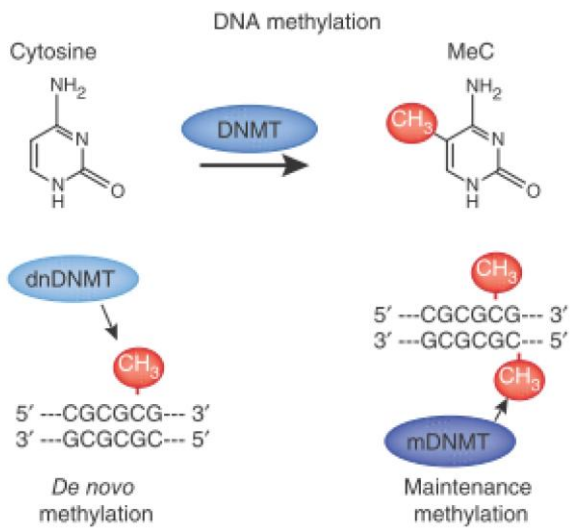


Figure 5 DNA methylations occurs at cytosine residues by addition of a methyl group at the 5′ position on the pyrimidine ring by a DNMT. Two types of DNMTs initiate DNA methylation. *De novo* DNMTs methylate non-methylated cytosines and maintenance DNMTs methylate hemi-methylated DNA on the complementary strand. Modified from Day and Sweatt, 2010.

1.5.2 Regulation of Transcription through Histone Modifications

The histones assembling the nucleosomal core hold the ability to modify the structure of chromatin, thus permitting transition from the dense heterochromatin to the active euchromatin and vice versa. Structural changes occur through modifications of the N-terminal tail of histones. These modifications include

acetylation, methylation, phosphorylation, ubiquitination, and sumoylation and are often present in combinations. It was believed that histone modifications induce either activation or repression of genes. However, modification domains which acts bivalently have recently been found, consequently proposing the idea that any modification will be able to activate or repress gene transcription under different circumstances. (Kouzarides, 2007) Though in relation to gene transcription the modifications can still be roughly divided into two different groups of modifications; 1) acetylation, methylation, phosphorylation, and ubiquitination, which are related to active gene transcription and 2) methylation, ubiquitination, sumoylation, and deimination, which are associated to repression of gene transcription. (Kouzarides, 2007) One of the best characterized modifications is acetylation, which arises at one or more lysine residues. Addition of acetyl groups are catalyzed by Histone Acetyltransferases (HAT) and removed by Histone Deacetylases (HDAC). As HATs neutralize the positive charge, and thereby weakens the interaction between the histone tails and the DNA. On the other hand when HDACs remove the acetyl group from lysine, a positive charge is restored resulting in a condensation of the negatively charged DNA, thus preventing transcription of genes. (Weaver, 2009)

Induction of chromatin modifications by ECS seems to regulate expression of cognitive- and plasticity related genes. Acute ECS induces acetylation of H4 while chronic ECS increases H3 acetylation, substantiating that H4 acetylations represent brief and dynamic changes and H3 acetylations mark stable and chronic events. All though the underlying mechanisms of ECS on histone modifications remain poorly understood, modifications of histones seem to be indispensable part of the search for novel treatments of depression. Moreover, current chronic antidepressants have been found to induce H3 methylation, resulting in increased transcription by mechanisms which are not clear. (Tsankova et al. 2007)

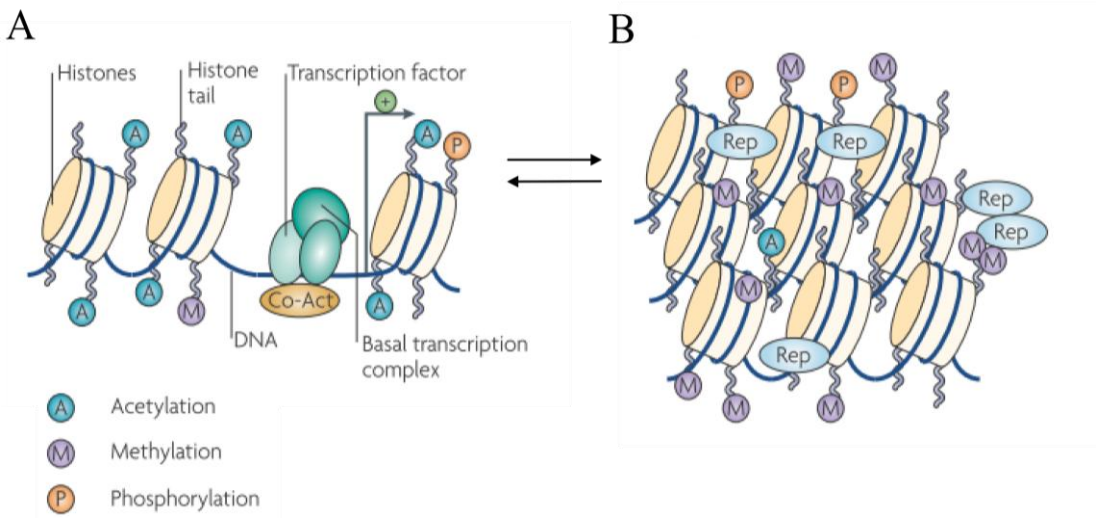


Figure 6 Chromatin is either at a transcriptionally active state or at a repressed state. The chromatin structure is dynamic and modifications determine the state of chromatin A) Transcription of genes is active and chromatin is called euchromatin. Histone modifications associated with euchromatin is e.g. acetylation, methylation and phosphorylation, transcription factors has easy access to the DNA. B) DNA is densely coiled around the histone octamer preventing transcription of genes. The chromatin is referred to as heterochromatin. Repressed DNA is mainly related to methylations. Modified from Tsankova et al. 2007.

2. Thesis Rationale

The aim of this thesis is to investigate the effect induced by MS and/or CMS on genes relevant for the aetiology of depression. Furthermore, the aim is to examine the methylational status of GR exon 1_7 in animals exposed to MS and/or CMS. Lastly should an optimization of a Native Chromatin Immunoprecipitation protocol be a future tool to investigate epigenetic changes associated with promoter regions.

This is accomplished using an animal model of depression established at the Center for Psychiatric Research, Risskov, Denmark by Kim Henningsen and Ove Wiborg. Through the animal model, 5 different experimental groups are reared; 1) a control group which is left unchallenged; 2) a group in which the animals are only exposed to MS, the animals of this group have an anhedonic phenotype; 3) a group where the animals are exposed to CMS, the animals in this group are also anhedonic; 4) a group where the animals are exposed to both MS and CMS, these animals have an anhedonic pheontype; 5) a group where the animals are exposed to both MS and CMS but these animals have a resilient pheontype. The 5 different animal groups allows for investigation of the difference between the MS and the CMS paradigm, and furthermore, for the difference between an anhedonic phenotype and a resilient phenotype when exposed to both MS and CMS.

3. Experimental Procedures

3.1 Materials

3.1.1 Instruments

Centrifuge MR23i (Thermo Scientific, USA)
Dialysis tubes, MWCO 6-8000 (Spectrum Laboratories Inc, USA)
Dri-Block™ DB-2A (Holme & Halby Techne, Denmark)
Dynabeads, 30 mg/mL (Invitrogen, USA)
Electrophoresis Cell Model Run One (Embi Tec, USA)
Eppendorf Centrifuge 5403R (Eppendorf, Germany)
Eppendorf Thermomixer Comfort (Eppendorf, Germany)
Intelli-Mixer RM-2L (Elmi, Latvia)
Kodak Image Station 4000MM PRO (Carestream Health Inc, USA)
KS 501 digital Laboratory Shaker (IKA* Works, USA)
Magnetic rack (Invitrogen, USA)
Magnetic stirrer Big Squid [Froggy] (IKA* Works, USA)
Microspin FV-2400 (Saveen Werner, Sweden)
Mx3000P™ QPCR System (Stratagene, USA)
NanoPhotometer™ (IMPLEN, Germany)
Petri Dishes, 60 x 15mm (Sarstedt, Germany)
TF-20M UV fluorescent table (Vilber Lourmat, Germany)
Thermal Cycler 2720 (Applied Biosystems, USA)
T10 basic Disperser/Homogenizer workcenter (IKA* Works, USA)
Vacuubrand Type RD8 (GMBH, Germany)
VCX-130 Ultrasonic Processor (Sonics, Vibra-Cell, USA)
Veriti™ 96-Well Thermal Cycler (Applied Biosystems, USA)
VR-1 Vacuum Evaporator (Heto-Holten, Denmark)
Water Thermostat TW-2 (Elmi, Latvia)
13 mL Reagent and Centrifuge Tube (Sarstedt, Germany)

3.1.2 Kits, Enzymes, Antibodies, and Competent Cells

Anti-Acetylated Histone H4 Antibody (Merck-Millipore, USA)
AllPrep DNA/RNA Mini Kit (Qiagen, Netherlands)
Brilliant II SYBR Green QPCR Master Mix (Stratagene, USA)
DNase I, 1u/µL (Fermentas, USA)
DreamTaq™ Green PCR Master Mix (Fermentas, USA)
EpiTect Bisulfite Kit (Qiagen, Netherlands)
GeneJET™ Plasmid Miniprep Kit (Fermentas, USA)
InsTAclone™ PCR Cloning Kit (Fermentas, USA)
MiniElute Gel Extraction Kit (Qiagen, Netherlands)
Micrococcal Nuclease (Sigma-Aldrich, USA)
Rabbit Serum (Invitrogen, USA)
Protease Inhibitor Cocktails, complete mini EDTA-free (Roche Diagnostics, Switzerland)

Proteinase K, 18.9 mg/mL (Fermentas, USA)
RevertAid™ Premium First Strand cDNA Synthesis Kit (Fermentas, USA)
RNase A, 10 mg/mL (Fermentas, USA)
ROX (Stratagene, USA)
Subcloning Efficiency™ DH5α™ Competent Cells (Invitrogen, USA)
TrueStart™ Hot Start Taq DNA Polymerase (Fermentas, USA)

3.1.3 Chemicals

Acetic acid (Sigma-Aldrich, USA)
Ampicillin Sodium Salt, (Sigma-Aldrich, USA)
β-mercaptoethanol (Invitrogen, USA)
Bovine Serum Albumin/BSA, 10 µg/mL (New England Biolabs, USA)
$CaCl_2$ (Sigma-Aldrich, USA)
Dithiothreitol/DTT (Sigma-Aldrich, USA)
EDTA, 50 mM (Fermentas, USA)
Ethanol 99.9% (Kemetyl A/S, Denmark)
Ethidium Bromide, 10 mg/mL (Sigma-Aldrich, USA)
GeneRuler™ 100 bp DNA ladder (Fermentas, USA)
GeneRuler™ 1 kb DNA ladder (Fermentas, USA)
Glucose (Sigma-Aldrich, USA)
HCl (Sigma-Aldrich, USA)
Igepal NP-40 (Sigma-Aldrich, USA)
KCl (Sigma-Aldrich, USA)
λ DNA (Fermentas, USA)
LB-Agar (Merck-Millipore, USA)
LiCl (Bie & Berntsen A/S, Denmark)
$MgCl_2$ (Sigma-Aldrich, USA)
NaCl (Sigma-Aldrich, USA)
NaOH (Sigma-Aldrich, USA)
Phenol:chloroform:IAA (Invitrogen, USA)
Potassium acetate (Sigma-Aldrich, USA)
SeaKemR LE Agarose (Lonza, Switzerland)
Sodium Bicarbonat/Na-HCO_3 (Sigma Aldrich, USA)
Sodium Butyrate/Na-Butyrate (Sigma Aldrich, USA)
Sodium Deoxycholate/Na-Deoxycholate (Sigma Aldrich, USA)
Sodium dodecyl sulfate/SDS (BioRad, USA)
Sucrose (Sigma Aldrich, USA)
Tris-Cl (Sigma Aldrich, USA)
Triton X-100 (Sigma Aldrich, USA)
Tryptone (Sigma Aldrich, USA)
Yeast Extract (Sigma Aldrich, USA)
6x Loading dye (Fermentas, USA)

3.1.4 Buffers

ChIP Dilution Buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-Cl (pH 7.9-8), 167 mM NaCl)
ChIP Equilibration Buffer (1.5 mL ChIP Dilution Buffer, 2 µg sonicated λ DNA, 2 mg Bovine Serum Albumin)
ChIP Lysis Buffer (50 mM Tris-Cl (pH 7.9-8), 10 mM EDTA, 1% SDS, Protease Inhibitors)
Dialysis-Lysis Buffer (1 mM Tris-Cl (pH 7.5), 0.2 mM EDTA, 5 mM Na-Butyrate, Protease Inhibitors)
Elution Buffer (1 % SDS, 0.1 M Na-HCO_3)
EnzNuclei Preparation Buffer I (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM Na-Butyrate, 0.1 mM EDTA, 15 mM Tris-Cl (pH 7.5), 0.5 mM DTT, Protease Inhibitors)
EnzNuclei Preparation Buffer II (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 5 mM Na-Butyrate, 0.1 mM EDTA, 15 mM Tris-Cl (pH 7.5), 0.5 mM DTT, 1 % Triton X-100, Protease Inhibitors)
High Salt Wash Buffer (500 mM NaCl, 0.1% SDS, 1 % Triton X-100, 2mM EDTA, 20 mM Tris-Cl (pH 7.9-8))
LiCl Wash Buffer (0.25 M LiCl, 1 % Igepal (NP-40), 1 % Na-Deoxycholate, 1 mM EDTA, 10 mM Tris-Cl (pH 7.9-8))
Low Salt Wash Buffer (150 mM NaCl, 0.1% SDS, 1 % Triton X-100, 2mM EDTA, 20 mM Tris-Cl (pH 7.9-8))
MNase Digestion Buffer (0.32 M sucrose, 50 mM Tris-Cl (pH 7.5), 4 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Na-Butyrate, Protease Inhibitors)
Plasmid Preparation Buffer I (50 nM glucose, 25 nM Tris-Cl (pH 8), 10 nM EDTA (pH 8), 10 ng/ml RNase A)
Plasmid Preparation Buffer II (0.2 N NaOH, 1% SDS)
Plasmid Preparation Buffer III (60 mL 5 M potassium acetate, 11.5 mL glacial acetic acid, 28.5 mL H_2O)
SOC Medium (Laboratory stock; 20 g Tryptone, 5 g Yeast Extract, 0,5 g NaCl, 10 mL 250 nM KCl, 0.2 mL 5 N NaOH, 18 mL 20 % glucose solution, 5 mL 2 M $MgCl_2$)
TAE Buffer (Laboratory stock: 40 mM Tris, 20 mM acetic acid and 1 mM Na_2EDTA)
TE Buffer (10 mM Tris-CL (pH 7.5), 1 mM EDTA)
Tubing Preparation Buffer I (2 % Na-HCO_3,1 mM EDTA)
Tubing Preparation Buffer II (1 mM EDTA)
SoniNuclei Preparation Buffer I (0.4 mM sucrose, 10 mM Tris-Cl (pH 7.9-8), 5 mM β-Mercaptoethanol, Protease Inhibitors)
SoniNuclei Preparation Buffer II (0.25 mM sucrose, 10 mM Tris-Cl (pH 7.9-8), 1% Triton X-100, 10 mM $MgCl_2$, 5 mM β-Mercaptoethanol, Protease Inhibitors)

3.2. Animals

The *in vivo* chronic mild stress (CMS) and maternal separation (MS) models were carried out at the Center for Psychiatric Research in Aarhus, Denmark by Kim Henningsen and Ove Wiborg. The animals used were outbred Wistar rats from Taconic M&B in Ry, Denmark. The rats has unlimited access to food and water, except when deprived as part of a stress parameter. A standard 12 hours light/dark cycle, with light on from 6 a.m. to 6 p.m., was normally followed except during periods of stress induction. The pups used throughout this experiment were only male.

3.2.1 Maternal Separation

A number of pups were chosen for MS protocol just after birth. According to the protocol these pups were separated from the mother 180 consecutive min. daily from 9:00 am - 12:00 am. The separation protocol lasted from postnatal day 2 to postnatal day 14, both days included. After separation the pups either entered the CMS paradigm or were left unchallenged. 6 pups were selected for a MS group, 12 pups were

used in the CMS protocol, and a small amount of pups were excluded from the experiment due to a too low sucrose ingest.

3.2.2 Chronic Mild Stress

Along with the pups exposed to the MS protocol another group of pups was kept 2 weeks under normal laboratory conditions. After the 6 weeks all pups were divided into two additional groups. One group which had not been exposed to MS but continued in a stress protocol and one group of pups exposed to separation and thereafter continued in the stress protocol. Furthermore, one group of pups was left unchallenged. Animals with a too low baseline sucrose ingest were excluded from further participation. The CMS protocol lasted seven weeks and consisted of seven different stress conditions each lasting 10 to 14 hours. One period of intermittent illumination, stroboscopic light, grouping, food or water deprivation, and two periods of soiled cage and no stress, and three periods of 45° cage-tilting. During the stress protocol sucrose intake was measured once a week and an animal was characterized as anhedonic if baseline sucrose intake declined by more than 30%, otherwise animals were characterized as resilient.

3.3 Tissue Preparation

Tissue preparation was carried out by Kim Henningsen at at Center for Psychiatric Research, Risskov, Denmark. The animals were decapitated at 15-16 weeks of age and the brains immediately dissected. The ventral part of both the left and right hippocampus were removed and snap-frozen. The tissue samples were stored at -80 °C until further use.

3.3.1 DNA and RNA Isolation

DNA and RNA were isolated from hippocampus of the right hemisphere using AllPrep DNA/RNA Mini Kit using the protocol Simultaneous Purification of Genomic DNA and Total RNA from Animal Tissues. Disruption and homogenization of tissue was done using a rotor stator homogenizer T10 basic Disperser/Homogenizer in 600 µL lysis Buffer RLT supplemented with 6 µL β-mercaptoethanol. Tissue samples weighed on average 25 mg. The rest of the procedure was performed according to manufacturer's protocol including the optional centrifugation step during RNA purification. RNA was eluted in 2x20 µL RNase free H_2O and DNA was eluted in 100 µL Buffer EB. DNA and RNA were immediately after treated with RNase and DNase, respectively.

After purification, DNA and RNA concentrations were measured and quality assessed by spectrophotometry using a NanoPhotometer™. Afterwards DNA and RNA from all tissue samples were run on an agarose gel, as described in section 3.4, to validate the quality of DNA and RNA.

3.4 Gel Electrophoresis

1 % agarose gel was made in 1x TAE Buffer, and Ethidium Bromide was added to a final concentration of 1 µg/mL. The mixture was added to a mold and allowed to settle. The gels were run in the Electrophoresis Cell Model Run One embedded in 1x TAE Buffer. To estimated product size 2 µL of either GeneRuler™ 100 bp DNA ladder or GeneRuler™ 1 kb DNA ladder was loaded onto the gel subsequently along with the product of interest. Samples were mixed with 6x Loading Dye prior to loading, unless they contained DreamTaq™ Green PCR Master Mix. For visualization of the gels a Kodak Image Station 4000MM PRO was used. The excitation filter was set to 530 nm and the emission filter 600 nm. If necessary, gels were visualized on a TF-20M UV fluorescent table.

3.5 Gene Expression Analysis

In order to compare the gene expression profile between the different groups of animals Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-qPCR) was performed. Primers used for RT-qPCR were designed using NCBI Primer designing tool Primer-BLAST. The following criteria were set for primer design; product size 100-150, melting temperature (Tm) should be min. 63 °C, opt. 65 °C, max. 67 °C, and Max T_m difference 2 °C, max self complementarity no more than 6, max 3' end complementarity no more than 3, and if possible primers should span an intron at the corresponding genomic DNA. All primer pairs were run at an annealing temperature of 60 °C. Most primers were kindly provided by Mads Dyrvig Johannesen or accessible through the research group, Laboratory of Neurobiology.

Table 1 Primers used in the gene expression analysis

Target gene:	Primer sequence:	Efficienciey:	NCBI ref. seq.
GAPDH exon 7	Fw: CATCAAGAAGGTGGTGAAGCA Rw: CTGTTGAAGTCACAGGAGACA	95.1 %	NM_017008.3
β-Actin 5'UTR	Fw: AAGGGACACCGTAGAGGGGTGGAGC Rw: CAGGAGCGTGCCCACGAGTGTCTAC	98 %	NW_047369.2
GR exon 1_7	Fw: AGGGAGCCTGGGAGAAGAGAAACTAA Rw: CTGGCCTGGGAGGGAAACCGAGT	105.8 %	AJ271870
GR exon 2/3	Fw: GGGCTCTGAACTTCCCAGGCCGGT Rw: GGTCCCGTGGCTGCTGACGAGC	103.4 %	NM_012576.2
Arc exon 2/3	Fw: GCTGGAGTCTTCAGAGCCAGGTGAA Rw: TGTGCAGGCAGCTTCAAGAGAGGAG	101.8 %	NW_047780.1
Rims1 exon 1/2	Fw: GCCCGACCTGAGCCACCTGACC Rw: TTCAGTGGTGGTTGATGGGGCTGGC	102.1 %	NM_052829.1
GAD1 exon 11/12	Fw: TGTCAATGCAACCGCAGGCACGACT Rw: GCGATGCTTCCGGGACATGAGCAGC	99.3 %	NM_017007.1
c-Fos exon 4	Fw: GGTCACAGAGCTGGAGCCCCTGTGC Rw: TCGTTGCTGCTGCTGCCCTTTCGGT	102.3 %	NW_047762.2
cJun exon 1/2	Fw: CCTCAACGCCTCGTTCCTCCAGTC Rw: CGTGAGAAGGTCCGAGTTCTTGGCT	105.8 %	NM_021835.3
FosB exon 1/2	Fw: GTCTTCGGTGGACTCCTTCGGCAGT Rw: GTCCTGGCTGGTTGTGATTGCGGTG	103,4 %	NM_001256509.1
JunB exon 1	Fw: GCTGTCAAGTACTGCCGGCCTCCTA Rw: GTGTCCGTATGGAGCAAGGGAGGCT	102 %	NM_021836.2

NPY exon 2/3	Fw: CATGGCCAGATACTACTCCGCTCTGCGA Rw: AGCCTTGTTCTGGGGGCATTTTCTGTGC	101.3 %	NM_001113357.1
Gal exon 3/4	Fw: GCTCGGGATGCCAACAAAGGAGAAG Rw: CCAGTGGTAACTCCCTCTTGCCTGT	110.4 %	NM_033237.1
DNMT3a exon 5/6	Fw: CGAAGGTTTACCCACCTGTGCCAGC Rw: ATGTAGCGGTCCACTTGGATGCCCA	104.6 %	NM_001003957.1
CREB exon 2/3	Fw: CAGTTCAAGCCCAGCCACAGATTGC Rw: CATGGACCTGGACTGTCTGCCCATT	106.4 %	NM_134443.1

3.5.1 Primer Optimization

Prior to usage of primers the annealing temperature was determined to see if the primers would be able to run properly at 60 °C. This was done by PCR using a 20 µL reaction mixture of 1x TrueStart™ Hot Start Taq buffer, 1 mM dNTP, 3.125 mM $MgCl_2$, 0.5 µM of each primer, and 1.25 u TrueStart™ Hot Start Taq DNA Polymerase. The amplification was carried out using a Veriti™ 96-Well Thermal Cycler programmed to 95 °C for 10 min., followed by 40 cycles of; 95 °C for 30 sec. and 60 °C for 30 sec. Afterwards the PCR products were visualized on a gel as described in section 3.4. Primers were also tested on genomic DNA as a control for specificity of the primers.

Efficiencie of the primer pairs were determined using a two-fold dilution series of total rat cDNA from one animal of the CMS group. Each dilution was run in triplicates in a 20 µL reaction with 1x Brilliant II SYBR Green QPCR Master Mix and 0.5 µM forward and reverse primer. The reactions were run using an Mx3000P™ QPCR System programmed to 95°C for 10 min., followed by 40 cycles of; 95 °C for 30 sec. and 60 °C for 30 sec. This was followed by a 25 min. melting curve program starting at 55 °C and ending at 95 °C.

3.5.2 cDNA Synthesis

RNA isolated from the right ventral hippocampus was used for cDNA synthesis using RevertAid™ Premium First Strand cDNA Synthesis Kit. cDNA was synthesized according to manufacturer's recommendations using the protocol for RT-qPCR. Equal amount of RNA (60 ng) was used for all cDNA syntheses. The reverse transcriptase reaction was run in a Veriti™ 96-Well Thermal Cycler at 25 °C for 10 min., 50 °C for 30 min., and 85 °C for 5 min. The cDNA was diluted 1:20 with H_2O and used immediately after or stored in aliquots at -20 °C until further use.

3.5.3 Quantitative Reverse Transcriptase Polymerase Chain Reaction

RT-qPCR was performed for all genes of interest using β-Actin as a reference gene. 10 µL of cDNA was amplified in a 20 µL reaction mixture of 1x Brilliant II SYBR Green QPCR Master Mix, 30 nM ROX and 0.5 µM of each primer. All cDNA samples and controls were run in duplicates. The amplification reactions were run in a Mx3000P™ QPCR System at 95 °C for 10 min., followed by 40 cycles of; 95 °C for 30 sec. and 60 °C for 30 sec. followed by a 25 min. melting curve program starting at 55 °C and ending at 95 °C. No-RT and NTC were included as a control for unspecific binding of primers and contamination of reagents.

3.6 Sodium Bisulfite Mapping

To investigate whether any changes seen through the expression analysis originates from methylational alterations at the promoter region of the gene, a sodium bisulfite mapping analysis was performed. The

genomic DNA isolated from all tissue samples was used for this analysis, which converts unmethylated cytosine residues to uracil and subsequently, via PCR amplification to thymidine. This process allows us to identify and investigate the methylational status of the gene promoter region. Primers used for amplification of bisulfite converted DNA were kindly donated from Mads Dyrvig Johannesen.

Table 2 Primers used for bisulfite sequencing PCR

Target gene:	Primer sequence:	Annealing:	NCBI ref. seq.
GR exon 1_7 promoter	Fw: TTTGTAGTTTTTTTGTTAGTGTGATAT Rw: ATTTCTTTAATTTCTCTTCTCCCAAA	48 °C	AJ271870

3.6.1 Bisulfite Treatment of Genomic DNA and Amplification

Bisulfite conversion of genomic DNA was done using an EpiTect Bisulfite kit, using the protocol for Sodium Bisulfite Conversion of Unmethylated Cytosines. The input DNA was 300 ng for each conversion and the reactions were run in a Veriti™ 96-Well Thermal Cycler with the reaction volume set to 100 µL. The procedure was done according to manufacturer's protocol with addition of a 2 min. incubation step with Buffer EB during elution. Elution was done using 2x20 µL Buffer EB. The eluted bisulfite converted DNA was diluted with 35 µL H_2O and used for PCR immediately after or stored at -20 °C.

Afterwards, bisulfite treated DNA was amplified by PCR using 10 µL template in a 20 µL reaction mixture. The mixture consisted of 1x TrueStart™ Hot Start Taq buffer, 1 mM dNTP, 3.125 mM $MgCl_2$, 0.5 µM of each primer, and 1.25 u TrueStart™ Hot Start Taq DNA Polymerase. The amplification was carried out using a Veriti™ 96-Well Thermal Cycler programmed to 95 °C for 5 min., followed by 35 cycles of; 95 °C for 1 min., 48 °C for 2.30 min., 68 °C for 1 min., and a final step of 68 °C for 5 min. The GAPDH primer pair used in the expression analysis was used as a negative control to validate the effectiveness of the bisulfite treatment. Moreover, water and wild-type genomic DNA were used as negative controls. After amplification the PCR products were run on an agarose gel as described in section 3.4.

3.6.2 Extraction from Agarose Gel

Subsequent to amplification of bisulfite converted DNA, the samples suitable for further processing were extracted from the gel. The samples suitable for further processing met following criteria: water control should be negative, wild-type genomic DNA should be negative, GAPDH negative control should be negative, and the actual band size should resemble the expected band size. DNA bands were cut out using a TF-20M UV fluorescent table and the DNA extracted by means of MinElute Gel Extraction Kit using the protocol MinElute Gel Extraction Kit Protocol using a Microcentrifuge. The protocol was followed according to manufacturer's recommendations and DNA was eluted using 10 µL Buffer EB. DNA concentration was measured on a Nanodrop ND-1000 spectrophotometer. Extracted bisulfite treated DNA was immediately after used for ligation.

3.6.3 Cloning Procedure

Ligation of extracted PCR products was carried out using InsTAclone™ PCR Cloning Kit according to manufacturer's protocol. The vector:insert ratio in the ligation reaction was 1:3, and the reaction time was

extended to overnight at 4 °C. Ligated DNA was used immediately after for transformation of E.coli or stored at -20 °C.

Subcloning Efficiency™ DH5α™ Competent Cells were used for transformation of ligated PCR products. The competent cells were thawed on ice and carefully mixed by inversion. For each ligated PCR product 50 µL of competent cells were used. The cells were mixed with 2.5 µL of ligation reaction and incubated on ice for 30 min. After incubation the mixture was heat shocked at 42 °C for 20 sec. using a Water Thermostat TW-2 and thereafter cooled on ice for 2 min. Next 950 µL SOC medium was added and the mixture was incubated at 37 °C for 1 h. at 225 rpm on a KS 501 digital Laboratory Shaker. Finally, samples were centrifuged using a Eppendorf Centrifuge 5430R at 5000 *g* for 1 min, 900 µL of the supernatant was discarded, and the remaining 100 µL was mixed by shaking carefully. The competent cells were plated onto agar plates and incubated at 37 °C overnight.

3.6.4 Colony Polymerase Chain Reaction

After incubation of competent E.coli cells overnight, 12 colonies were selected and screened for containment of target DNA using colony PCR. A reaction mixture of 20 µL containing 10 µL H_2O, 1x DreamTaq™ Green PCR Master Mix and 1 µM of each M13 primer was prepared and mixed with a small amount from one colony. The colony PCR was run in the Veriti™ 96-Well Thermal Cycler programmed to 95 °C for 5 min., followed by 30 cycles of; 95 °C for 30 sec., 60 °C for 30 sec., 72 °C for 30 sec., and a final step of 72 °C for 5 min. Afterwards, the colonies were visualized on a agarose gel as described in section 3.4.

3.6.5 Extraction of Plasmids

Colonies positive for the correct insert were selected for further processing. These colonies were transferred to a 13 mL Reagent and Centrifuge Tube containing 2 mL LB-medium with 100 µg/mL Ampicillin. The tubes were incubated at 37 °C overnight at 200 rpm on a KS 501 digital Laboratory Shaker. Plasmids were extracted after incubation using either GeneJet™ Plasmid Miniprep kit according to manufacturer's protocol or using the Alkaline Lysis Small-Scale Preparations of Plasmid DNA (method available in: Molecular Cloning, A Laboratory Manual). Briefly, the method is as follows; after incubation overnight in LB media the bacteria were centrifuged using a Eppendorf Centrifuge 5430R at max speed for 30 sec. at 4 °C. The supernatant was discarded and the dry pellet was resuspended in 100 µL ice-cold Plasmid Preparation Buffer I by vigorous vortexing using a Microspin FV-2400. Thereafter, bacteria were incubated 10 min. at 37 °C and 200 µL of freshly made Plasmid Preparation Buffer II was added. The mixture was mixed by inversion for 10 sec. and chilled on ice. 150 µL ice cold Plasmid Preparation Buffer III was added and the mixtures were inverted for 10 sec. Mixtures were incubated on ice for 3-5 min. and then centrifuged at maximum speed for 5 min. at 4 °C. The supernatant was transferred to a new tube. One volume of phenol:chloroform:IAA was added to extract plasmids from the residual. The mixture was briefly vortexed using the Microspin FV-2400 and centrifuged by the Eppendorf Centrifuge 5430R at 12000 g for 2 min. at 4 °C. The supernatant was added to a fresh tube and the DNA was precipitated using 2 volumes of ethanol at RT and allowed to settle for 2 min. DNA was centrifuged using the Eppendorf Centrifuge 5430R at 12000 g for 2 min. at 4 °C and the supernatant was carefully discarded. The tubes were positioned inverted on a paper towel and allowed to dry for 5 min. before adding 1 mL of ice-cold 70% ethanol to rinse the pellet. Ethanol was cautiously removed and pellet allowed to dry for 10 min. before redissolving it in 50 µL 1 M Tris-Cl (pH 8). For each sample the concentration of DNA was determined using a NanoPhotometer™. 1.5 µg of DNA was dried using a Vacuubrand Type RD8 connected to a VR-1 Vacuum

Evaporator. The dried bisulfite treated DNA was sent to Beckman Coulter Genomics, United Kingdom, for sequencing.

3.7 Native Chromatin Immunoprecipitation

In order to investigate potential histone modifications related to gene promoter regions, an optimization of a native chromatin immunoprecipitation (NChIP) protocol was initiated. Two methods were used to fragmentize the chromatin; sonication or enzymatic digestion with MNases.

3.7.1 Preparation of Dialysis Tubes

Dialysis tubes (10 kDa pore width) of 15 cm in length were boiled in for 10 min. in 0.5 L of Tubing Preparation Buffer I and rinsed twice in distilled H_2O. The tubes were then boiled for 10 min. in Tubing Preparation Buffer II. After boiling the tubes were allowed to cool down and stored imbedded in Tubing Preparation Buffer II at 4 °C. Before usage the tubes were carefully rinsed in distilled H_2O inside and outside.

3.7.2 Preparation of Dynabeads Protein A

The needed amount of Dynabeads was washed and prepared prior to usage. The beads were washed in 3x1 mL ChIP Dilution Buffer and incubated on Intelli-mixer RM-2L rotator in 1.5 mL ChIP Equilibration Buffer overnight at 4 °C. After incubation the beads were washed in 3x1 mL ChIP Dilution Buffer, the beads were then resuspended in a volume corresponding to the amount of samples x50 µL ChIP Dilution Buffer. The beads were used immediately after.

3.7.3 Fractionation through Sonication Procedure

One frozen rat hippocampus was homogenized in 10 mL of ice cold SoniNuclei Preparation Buffer I. Homogenization was done using a T10 basic Disperser/Homogenizer in 4x10 sec. starting at step one for 10 sec. followed by a stepwise increment in speed until step four. The homogenate was then centrifuged at 3000 *g* for 20 min. at 4 °C using Centrifuge MR23i. The supernatant was discarded and pellet was resuspended in 1 mL ice cold SoniNuclei Preparation Buffer II. The mixture was centrifuged at 12000 *g* for 10 min. at 4 °C, the supernatant was discarded, and pellet was resuspended in 300 µL ChIP Lysis Buffer. The chromatin Lysis Buffer solution was incubated on ice for 30 min. and 2.7 mL ChIP Dilution Buffer was added. In order to fractionize the chromatin, the mixtures were sonicated while on ice in 6x10 sec. at 10 % or 20 % output power using a VCX-130 Ultrasonic Processor. The sonicated nuclei were centrifuged using Centrifuge MR23i at maximum speed for 10 min. at 4 °C and the supernatant was collected and aliqouted into new tubes. Each aliquot contained 500 µL suspension.

Quality Control of Chromatin

The sonicated chromatin was mixed with 10 µL 0.5 mM EDTA, 20 µL 1M Tris-Cl (pH 6.5-6.8), and 1.2 µL 18.9 mg/mL Proteinase K and incubated for 3 h at 45 °C using a Thermal Cycler 2720. After incubation ChIP DNA Clean and Concentrator™ Kit was used to recover DNA. Extraction of DNA was performed according to manufacturer's recommendations. The quality and fraction size of the extracted DNA was assessed by gel electrophoresis as described in section 3.4.

3.7.4 Fractionation through Enzymatic Digestion Procedure

One frozen rat hippocampus was homogenized in 10 mL of ice cold EnzNuclei Perparation Buffer I. Homogenization was done using a T10 basic Disperser/Homogenizer in 4x10 sec. starting at step one for 10

sec. followed by a stepwise increment in speed until step four. The homogenate was then centrifuged at 3000 *g* for 20 min. at 4 °C using a Eppendorf Centrifuge 5430R. The supernatant was discarded and pellet was resuspended in 2 mL ice cold EnzNuclei Perparation Buffer I with addition of 2 mL ice cold EnzNuclei Perparation Buffer II. The mixture was gently mixed and centrifuged at 10000 g for 20 min at 4 °C. The supernatant was discarded and pellet was resusdended in 1 mL of MNase Digestion Buffer. The chromatin MNase Digestion Buffer solution was then centrifuged at 10.000 *g* for 20 min at 4 °C, the supernatant was discarded, and pellet was resuspended in 1 mL MNase Digestion Buffer. Each tube was split into two new tubes of 500 µL suspension.

To each tube of chromatin and MNase Digestion Buffer 33 or 75 units of MNase enzyme were added and mixed gently. The tubes were then incubated in a water bath at 37 °C using a Water Thermostat TW-2 for 3, 4, or 10 min. Rapidly thereafter 20 µL of 20 mM EDTA was added and samples were chilled on ice. The MNase digested nuclei were centrifuged at 10000 rpm for 10 min. at 4 °C and the supernatant containing the first soluble fraction of chromatin, the S1 fraction, was transferred to a new tube and stored at -20 °C for further use. Pellet was resuspended in 500 µL Dialysis-Lysis Buffer and transferred to a dialysis tube. Dialysis was performed overnight on an IKA Big-Squid magnetic stirrer at 4 °C in 2 L Dialysis-Lysis Buffer. After dialysis the dialyzed suspension, the S2 fraction, was centrifuged at 10000 rpm for 10 min. at 4 °C, the supernatant was transferred to a new tube. Pellet was resuspended in 50 µL Dialysis-Lysis Buffer. This was the P fraction.

Quality Control of Chromatin

After fractionation S1, S2 and P fractions were run in a 1.2 % agarose gel containing 1x TAE Buffer and 0.1 % SDS. The gel was stained with 20 µg Ethidium Bromide in 500 mL H_2O for 30 min., and rinsed with H_2O for 15 min. Gels were visualized as described in section 3.4. The S1 and S2 fraction were combined for further processing and split into two aliquots of 500 µL each.

3.7.5 Chromatin Immunoprecipitation

After fractionation of chromatin either by enzymatic digestion or by sonication, antibodies were added to the mixtures. The antibodies used were anti- Acetylated Histone H4 and Rabbit Serum for mock control. One tube was left untouched for input control. The mixtures of fractionized chromatin and antibody were incubated overnight on a IKA Big-Squid magnetic stirrer at 4 °C, thereafter 50 µL Dynabeads were added to all mixtures containing antibody or mock control, input control was left untouched, and all tubes were incubated for 3 h on a Intelli-Mixer RM-2L rotator at 4 °C. The samples containing Dynabeads were then washed on the rotator for 5x10 min. at 4 °C using 1 mL of different buffers. The order and number of washes were as follows:

1x Low Salt Wash Buffer
1x High Salt Wash Buffer
1x LiCl Wash Buffer
2x TE Buffer

Between each wash the beads were pelleted using a magnetic rack. Immune complexes were then eluted by incubation at 1000 rpm for 15 min. at 65 °C using a Thermomixer. Elution was performed twice using 2x250 µL freshly made Elution Buffer. 100 µL of input control was mixed with 400 µL Elution Buffer.
The eluted immune complexes were mixed with 10 µL 0.5 mM EDTA, 20 µL 1M Tris-Cl (pH 6.5-6.8), and 1.2 µL 18.9 mg/mL Proteinase K and incubated for 3 h at 45 °C using a Thermal Cycler 2720.

3.7.6 Extraction of DNA

ChIP DNA Clean and Concentrator™ Kit was used to recover immunoprecipitated DNA. Extraction of DNA was performed according to manufacturer's protocol. For each 100 µL was mixed with 500 µL ChIP DNA Binding Buffer, DNA was eluted using 30 µL of Elution Buffer. DNA was used immediately after or stored at -20 °C.

3.7.7 Native Chromatin Real-Time Quantitative Polymerase Chain Reaction

After extraction of precipitated DNA RT-qPCR was performed using primers specific for β-Actin promoter. All DNA samples were run in duplicates. 10 µL of DNA was amplified in a 20 µL reaction mixture of 1x Brilliant II SYBR Green QPCR Master Mix and 0.5 µM of each primer. The amplification reaction was run in an Mx3000P™ QPCR System at 95 °C for 10 min., followed by 40 cycles of; 95 °C for 30 sec. and 60 °C for 30 sec. followed by a 25 min. melting curve program starting at 55 °C and ending at 95 °C. No-RT was used as a control for contamination of reagents and unspecific binding of primers.

3.8 Data analysis

3.8.1 Gene Expression Analysis

The expression of each gene of interest was normalized to the reference gene β-Actin. The expression was calculated using following formula, where E equals the efficiencey of the primer:

$$\text{Gene expression} = \frac{(1 + \text{E}_{\text{Target Gene}})^{-\text{CQ}_{\text{Target Gene}}}}{(1 + \text{E}_{\text{Reference Gene}})^{-\text{CQ}_{\text{Reference Gene}}}}$$

3.8.2 Statistical Analysis

Statistical analysis of gene expression was done using GraphPad Prism 5. A normal distribution is assumed as al data derives from naturally occurring events. Data was analysed using a one-way ANOVA. A Newman-Keuls test was used to test for multiple comparison. Statistical significance was assigned to p values < 0.05.

3.8.3 Sodium Bisulfite Mapping

Data received from Beckman Coulter Genomics was analyzed using BiQ Analyzer software 2.0. The sequences of the bisulfite treated DNA were aligned to determine possible group differences.

4. Results

In the following section the results of the project will be presented. First the results of the gene expression analysis will be presented. Based on the results from the expression analysis and previous data obtained in our laboratory (Henningsen et al. 2012) the promoter region of the GR exon 1_7 was screened for methylated CpG sites. Lastly the optimization of the NChIP protocol will be presented.

4.1 Gene Expression Analysis

The gene expression analysis was performed on a total of five experimental groups each containing six animals. The five groups were: 1) control group which was left unchallenged; 2) a group in which the animals had been exposed to only MS, the animals of this group had an anhedonic phenotype; 3) a group where the animals were exposed to the CMS protocol, the animals in this group were also anhedonic; 4) a group where the animals had been exposed to both MS and CMS, these animals had an anhedonic pheontype; 5) a group where the animals had been exposed to both MS and CMS, these animals had a resilient pheontype. Figure 7 shows an overview of the experimental groups.

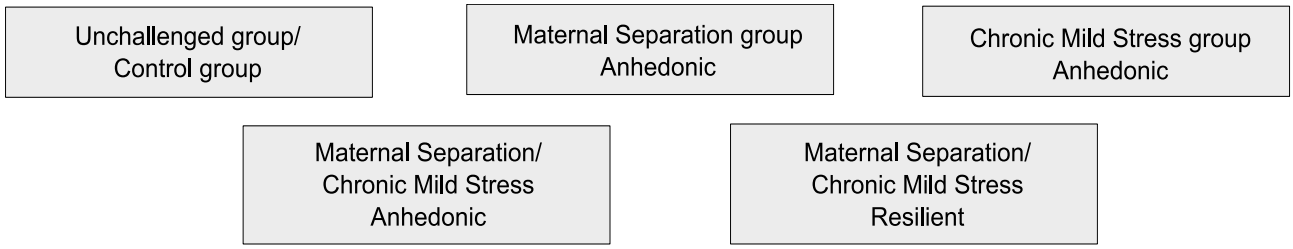


Figure 7 The five groups of animals.

4.1.1 Genes Included in Gene Expression Analysis

Maternal care has been proven to influence cognitive behaviour, as children who were subject to early adverse events might risk developing psychopathologies like depression. Impaired maternal care like decreased licking and grooming or separation of pups from mother has especially been seen to alter functions of hippocampus e.g. regulation of HPA-axis, memory formation or learning. (Zhang et al. 2010)(Zhang et al. 2004)(Marais et al. 2008) In this study the expression of genes involved in functions of the hippocampal structure of rat pups from the five different experimental groups were investigated, see Figure 7. The actions of AP-1 complex subunits have been strongly implicated in duration and stabilization of LTP, a critical process for memory and learning. Increases of c-Fos and c-Jun as high as 4- to 5-fold has been observed subsequent to a learning inducing behaviour task. (Herdegen and Leah, 1998) Thus, in order to determine whether certain subunits of the AP-1 complex is under the influence of maternal care and/or induction of mild stressors , the expression of c-Jun, c-Fos, FosB, and JunB were investigated in this study. Gal and NPY, two down-stream targets of the AP-1 complex, were chosen for the gene expression analysis as well. The neuropeptide Gal has been proven to be involved in formation of both spatial memory and learning, as Gal has been suggested to down-regulate these two functions. (Kinney et al. 2009) NPY on the contrary has been shown to positively correlate to recovery of depressive behaviour. Administration of the neuropeptide NPY is proposed to regulate the HPA-axis response, whereby the stress response is decreased. (Cohen et al. 2012) Gal and NPY were therefore also included as spatial memory and learning and the regulation of HPA-axis is crucial functions of hippocampus.

The HPA-axis is also regulated by GR expression, and interestingly the promoter region of GR exon 1_7 is thought to be under influence of maternal behaviour. (Henningsen et al. 2012)(Weaver et al. 2004) Therefore it was decided to include GR exon 1_7 promoter. Further, it was decided to include analysis of the whole GR expression, as the GR spliced variants does not differ in function, though they are expressed in a tissue specific manner. The whole GR expression, by means of GR exon 2/3, would consequently establish the amount of the total receptor. (McCormick et al. 2000) Furthermore, it was decided to include the two plasticity related genes; Arc and Rims1α, which are both associated with changed neuroplasticity. Arc is related to late phase consolidation of LTP and Rims1α is through interaction with synaptic vesicle protein Rab3 essential for presynaptic long-term plasticity. (Kaeser et al. 2008)

CREB is another transcription factor which has been proven to be implicated in depressive behaviour and cognitive impairments like dysfunction of LTP dependent memory formation. (Nestler et al. 2002) Moreover, CREB is implicated in regulation of the AP-1-complex and expression of Arc and GR, which makes it an interesting target for our expression analysis. (Herdegen and Leah, 1998)(Lam et al. 2009)(Weaver et al. 2001) Lastly, analysis of DNMT3a and GAD1 expression was included. DNMT3a has like CREB been found to be implicated in cognitive impairments. A decrease in DNMT3a correlates with impaired long term memory formation and further in a decrease in plasticity related gene Arc. (Oliveira et al. 2012) The expression of the GABA-regulating protein GAD1 was analysed as it has been found that GAD1 expression is influenced by maternal care. Zhang et al. 2010 found that maternal care influences the GABAergic system by altering GAD1 promoter methylation.

It should be noted that the primers for the Gal gene had a very high efficienciey, see Table 1, which might disguise the actual Gal expression by means of amplification of excess products e.g. primer dimers. Therefore the results obtained for this gene is marked not completely reliable and may not reflect the actual expression of Gal. mRNA levels of the genes are normalised to β-Actin and data is depicted as percentage of mean of the control group ±SEM, and the control group is set to 100 %.

4.1.1 Subunits of the AP-1 Complex

Through this study the expression of four subunits of the AP-1 complex was investigated, these four were c-Fos, c-Jun, FosB, and JunB. Interestingly, the results shows that both CMS and MS influence the expression of the four subunits. Through the analysis of c-Fos expression it was seen that the CMS anhedonic rats had a significantly higher c-Fos expression compared to the control group (p-value; <0.05), see Figure 8. Opposing, the expression of c-Fos in the MS anhedonic group was slightly lower compared to the control group, even though this was not statistically significant. The same differences also accounted for subunits FosB and JunB, where the expression was significantly higher in the CMS anhedonic rats compared to the control group (p-values; <0.05, <0.001 respectively). Further the decrease in gene expression in the MS anhedonic rat group compared to the control group was also present in the analysis of FosB and JunB expression. The difference was statistically significant for JunB (p-value; <0.0001). Interestingly, the CMS anhedonic group had a significantly higher genetic expression of these three genes than the MS group, even though both animal groups had an anhedonic phenotype (p-values; c-Fos <0.01, FosB <0.01, JunB <0.01), see Figure 8.

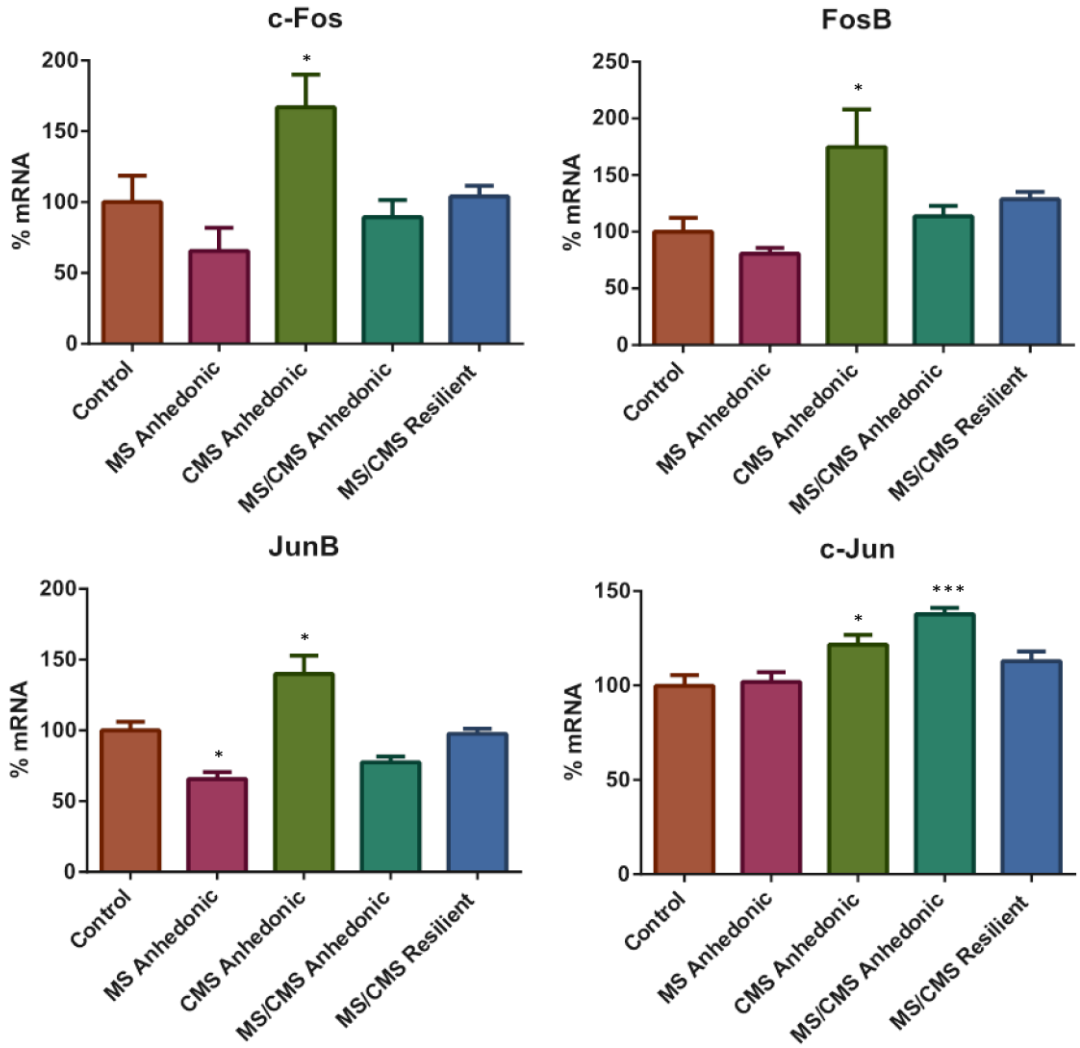


Figure 8 Expression of the subunits of AP-1 complex; c-Fos, FosB, JunB, and c-Jun. n=6 for all groups except the CMS anhedonic where n=5. Data is presented as percentage of mean of the control group ±SEM. The control group is set to 100 %. Significance levels shown on the figure are depicted as significant different from the control group. * represents P ≤ 0.05, and *** P ≤ 0.001

The expression of c-Fos in the CMS anhedonic group was furthermore significantly lower from both the MS/CMS anhedonic (p-value; <0.05) and the MS/CMS resilient group (p-value; <0.05). This also accounted for FosB where the CMS anhedonic group was significantly higher from the MS/CMS anhedonic group with a p-value of <0.05 and the CMS anhedonic group was significantly different from the MS/CMS resilient group with a p-value of <0.05. In the expression analysis of JunB the CMS anhedonic group had a statistically significant higher expression compared to the MS/CMS anhedonic group and the MS/CMS resilient group (p-values; <0.0001, <0.001 respectively), see Figure 8.

The expression of c-Fos, FosB and JunB was slightly lower in the MS anhedonic animal group compared to both the MS/CMS anhedonic and resilient group. However, this tendency was not significant. Among the two animal groups which had both been exposed to CMS and MS there was a tendency of a higher expression of c-Fos, in the MS/CMS resilient group when compared to the MS/CMS anhdonic group, see Figure 8. However, this tendency was not significant. This tendency was also present in the expression

analysis of both FosB and JunB, where the MS/CMS resilient group also had a slightly higher expression of the two genes compared to the MS/CMS anhedonic group.

The last AP-1 subunit c-Jun did not follow the expression pattern as described above. When comparing the control group to the MS anhedonic group the expression of c-Jun was more or less the same. On the contrary, the c-Jun gene expression of the CMS anhedonic group was significantly higher than the control group and the MS anhedonic group (p-values; <0.05, <0.05, respectively), see Figure 8. This increased expression in the CMS anhedonic group compared to the control group was also seen in the analysis of c-Fos, FosB and JunB expression. When comparing the MS/CMS anhedonic animal group to the MS/CMS resilient group it was observed that the genetic expression of c-Jun was higher in the anhedonic group (p-value; <0.01). Interestingly, this is the opposite of the expression pattern for the three other AP-1 subunits c-Fos, FosB, and JunB, when comparing these two animal groups, Figure 8.

4.1.2 Down-stream Targets of the AP-1 Complex

Two down-stream targets of the AP-1 complex were chosen for expression analysis to see if the gene expression pattern seen for the subunits would be recognizable in the targets. The targets chosen were; Gal and NPY, which both are neuropeptides and have an AP-1 consensus sequence embedded in their genetic code. Gal has been described to have a potent modulatory effect on the serotonergic system in hippocampus, which may contribute to depressive behaviour. (Ögren et al. 1998) The other down-stream target of the AP-1 complex NPY have been described to mediate positive regulation of the HPA-axis. (Cohen et al. 2012) Thus, making these two genes relevant targets of the AP-1 complex when considering the functions of the hippocampal structure.

When comparing the Gal expression in the control group to the MS anhedonic group it was seen that the genetic expression was lower in the MS group see Figure 9, this finding correspond to the results of the c-Fos, Fos, and JunB genes. Likewise, the expression in the CMS anhedonic group was higher than the expression in the MS group, which also was alike the pattern of the three subunits. The expression of Gal in the CMS anhedonic group was more or less the same as the expression in the control group. When comparing the MS/CMS resilient group to the MS/CMS anhedonic group, there was a tendency of a higher expression of Gal in the resilient group. Further was the Gal gene expression higher in the MS/CMS anhedonic and resilient group compared to the MS anhedonic and CMS anhedonic groups. However, none of this was statistically significant.

In the analysis of the NPY expression an increased was observed in both the MS and CMS anhedonic groups compared to the control group (p-values; <0.05, <0.01, respectively), see Figure 9. The increased expression in the CMS group compared to the control group also corresponds to the increase seen in the three AP-1 subunits c-Fos, FosB, and JunB. Furthermore, the expression of NPY was slightly higher in the CMS anhedonic group compared to the MS anhedonic group. However, this was not statistically significant. The gene expression of NPY was slightly higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group, this was not a statistically significant difference, see Figure 9. Furthermore, as it also accounted for Gal expression, the expression of NPY was higher in the MS/CMS anhedonic and resilient groups compared to the CMS anhedonic group. The expression was also increased in the MS/CMS anhedonic and resilient groups compared to the MS anhedonic group, this difference was statistically significant with p-values of <0.05 and <0.01, respectively.

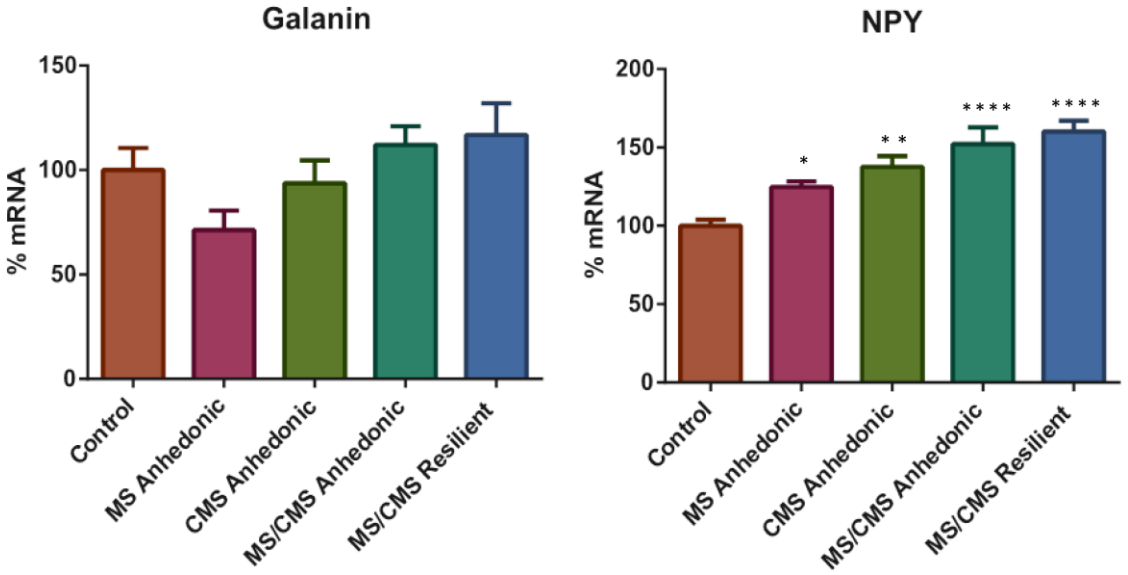


Figure 9 Expression of down-stream targets of the AP-1 complex Gal and NPY. n=6 for all groups except the CMS anhedonic where n=5. Data is presented as percentage of mean of the control group ±SE. The control group is set to 100 %. Significance levels shown on the figure are depicted as significant different from the control group. * represents P ≤ 0.05, ** P ≤ 0.01, and **** P ≤ 0.0001

Through the gene expression analysis of both Gal and NPY MS and CMS combined seemed to increase the gene expression, this opposes the tendency seen in the analysis of c-Fos, FosB, and JunB. The expression of Gal and NPY was higher in the MS/CMS anhedonic and the MS/CMS resilient groups compared to both the MS and CMS anhedonic groups.

4.1.3 Glucocorticoid Receptor

The GR is an important regulator of the HPA-axis responses and it has been found to be altered in depressive patients. Dysregulation of the HPA-axis may both result in neurotoxic damage due to sustained elevated cortisol levels and cognitive impairments amongst other things. (Nestler et al. 2002)(Weaver et al. 2001) Thus, GR serves as an interesting target in this model of MS and CMS exposure. Furthermore, it has been found that the GR promoter for exon 1_7 is under the influence of maternal behaviour and it has been postulated that this regulation might be mediated by epigenetic changes. (Weaver et al. 2001) Therefore, both GR and GR exon 1_7 were included in this expression analysis.

Interestingly, the overall expression of GR and the fraction of GR comprised of exon 1_7 showed two different expression patterns. The overall gene expression of GR, referred to as GR exon 2/3, did not seem to be influenced by either of the experimental groups, as the expression of GR exon 2/3 was more or less the same when comparing all animal groups, see Figure 10 GR exon 2/3. However, the expression of GR exon 1_7 varied among the different animal groups. It was seen that the expression of GR exon 1_7 was increased in the MS and the CMS anhedonic groups compared to the control group. Furthermore, the expression was higher in the MS anhedonic group compared to the CMS anhedonic group, see Figure 10. The MS/CMS anhedonic group showed a lower gene expression of GR exon 1_7 compared to the MS/CMS resilient group. Furthermore, the expression of GR exon 1_7 seems higher in the MS anhedonic group compared to both the MS/CMS anhedonic and resilient group, whereas the expression in the CMS

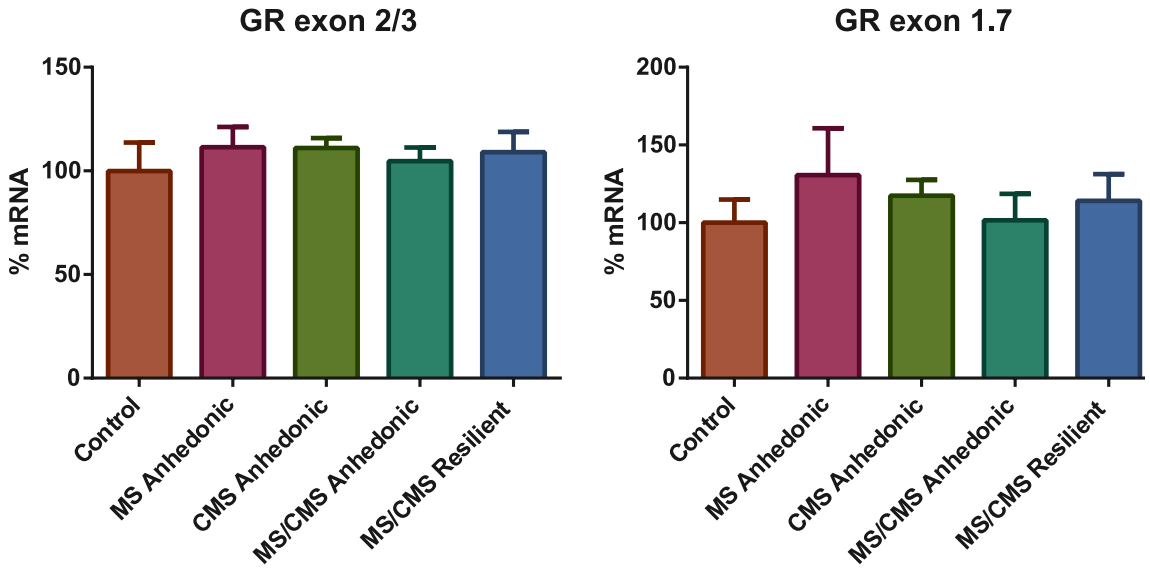


Figure 10 Expression of Glucocorticoid receptor exon 1_7 and 2/3. n=6 for all groups except the CMS anhedonic where n=5. Data is presented as percentage of mean of the control group ±SE. The control group is set to 100 %.

anhedonic group was more or less the same as the MS/CMS resilient group, but slightly higher than the MS/CMS anhedonic group. However, none of these differences were statistically significant.

4.1.4 Plasticity Related Genes

To investigate the influence of MS and CMS on plasticity related genes, Arc and Rims1α were included. Arc is a protein which has been associated with consolidation of both LTP and LTD, making Arc a regulator of synaptic homeostasis. (Coppens et al. 2011)(Bramham et al. 2008) Whereas Rims1α is related to long term plasticity at the synapse and to consolidation, which are processes essential for maintenance of cognitive behaviour. (Kaeser et al. 2008)(Kaeser and Südhof, 2005) Synapse plasticity, LTP and consolidation are all processes widely used in the hippocampal structure for e.g. memory and learning. Therefore, Arc and Rims1α are interesting targets of our gene expression analysis.

In the analysis of Arc expression it was seen that Arc was more widely expressed in the MS anhedonic and in the CMS anhedonic groups compared to the control group, see Figure 11. Further, it was seen that the expression was slightly higher in the CMS anhedonic group compared to the MS anhedonic group. However, none of these differences was statistically significant. It was additionally found that the expression of Arc in the MS/CMS resilient group was higher than in the MS/CMS anhedonic group. Interestingly, the expression was more or less the same in the MS anhedonic and in the MS/CMS resilient groups, see Figure 11 Arc. In the Rims1α analysis the expression was higher in the MS anhedonic group compared to both the control and the CMS anhedonic group, this different was not statistically significant. Interestingly, Rims1α was expressed in almost equal amounts in the control group and the CMS anhedonic group. Furthermore, the expression of Rims1α was slightly higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group, see Figure 11.

4.1.5 Other Genes

Three other genes were chosen for the expression analysis, these were CREB, DNMT3a and GAD1. CREB is a transcription factor which has been shown to be closely implicated with synaptic plasticity and LTP in the

hippocampus. (Nestler et al. 2002) Interestingly, some of the down-stream targets of CREB are AP-1-complex, Arc and GR. (Herdegen and Leah, 1998)(Lam et al. 2009)(Weaver et al. 2001) The methyltransferase DNMT3a was also included as it has been found that it is related to long term memory formation and plasticity related genes BDNF and Arc. Furthermore, DNMT3a is involved in cognitive impairments related to aging. (Oliveira et al. 2012) Lastly, the enzyme GAD1 was included in the expression analysis as it is involved in regulation of Glu/GABAergic system homeostasis. GAD1 has been proposed to be under the influence of maternal care, thus the alterations of the Glu/GABAergic systems seen in depression might be partially due to altered GAD1 expression. (Zhang et al. 2010)

Through the analysis of CREB, it was seen that the expression was higher in both the MS anhedonic and the CMS anhedonic group when compared to the control group, these differences was statistically significant (p-values; <0.05, <0.001, respectively). Additionally, the expression was increased in the CMS anhedonic group compared to the MS anhedonic group (p-value; <0.05). When comparing the MS/CMS anhedonic group to the MS/CMS resilient group the CREB expression was higher in the MS/CMS resilient group (p-value; <0.05). Moreover the MS anhedonic group showed a statistically significant decrease in expression compared to the MS/CMS resilient group (p-value; <0.001). The expression was also slightly higher in the MS/CMS anhedonic group compared to the MS anhedonic group, however this was not significant. Interestingly, CREB expression was more or less the same in the CMS anhedonic and the MS/CMS anhedonic groups, however the MS/CMS resilient group showed a higher expression compared to the CMS anhedonic group, see Figure 11. It seems as if CREB share the same tendencies with its down-stream target Arc, both genes were expressed more widely in both the MS and CMS anhedonic groups compared to the control group, and there was a higher expression in the CMS anhedonic group compared to the MS anhedonic group. Even further, the expression of both CREB and Arc were higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group see Figure 11 CREB and Arc.

Through the analysis of DNMT3a expression it was seen that DNMT3a was more widely expressed in the MS anhedonic group compared to both the control and CMS anhedonic groups. Whereas DNMT3a was more or less equally expressed in the CMS anhedonic group and the control group, see Figure 11 DNMT3a. Interestingly, the expression was decreased in the MS/CMS anhedonic group compared to the MS/CMS resilient group, and also compared to both the MS anhedonic and the CMS anhedonic groups. However, this was not statistically significant. The last gene included was GAD1 and in the expression analysis it was seen that GAD1 was expressed in almost equal amounts in the control group, the CMS anhedonic group, the MS/CMS anhedonic, and the MS/CMS resilient group. However, in the MS anhedonic group, the expression was slightly decreased compared to the other experimental groups, though this was not statistically significant, see Figure 11 GAD1.

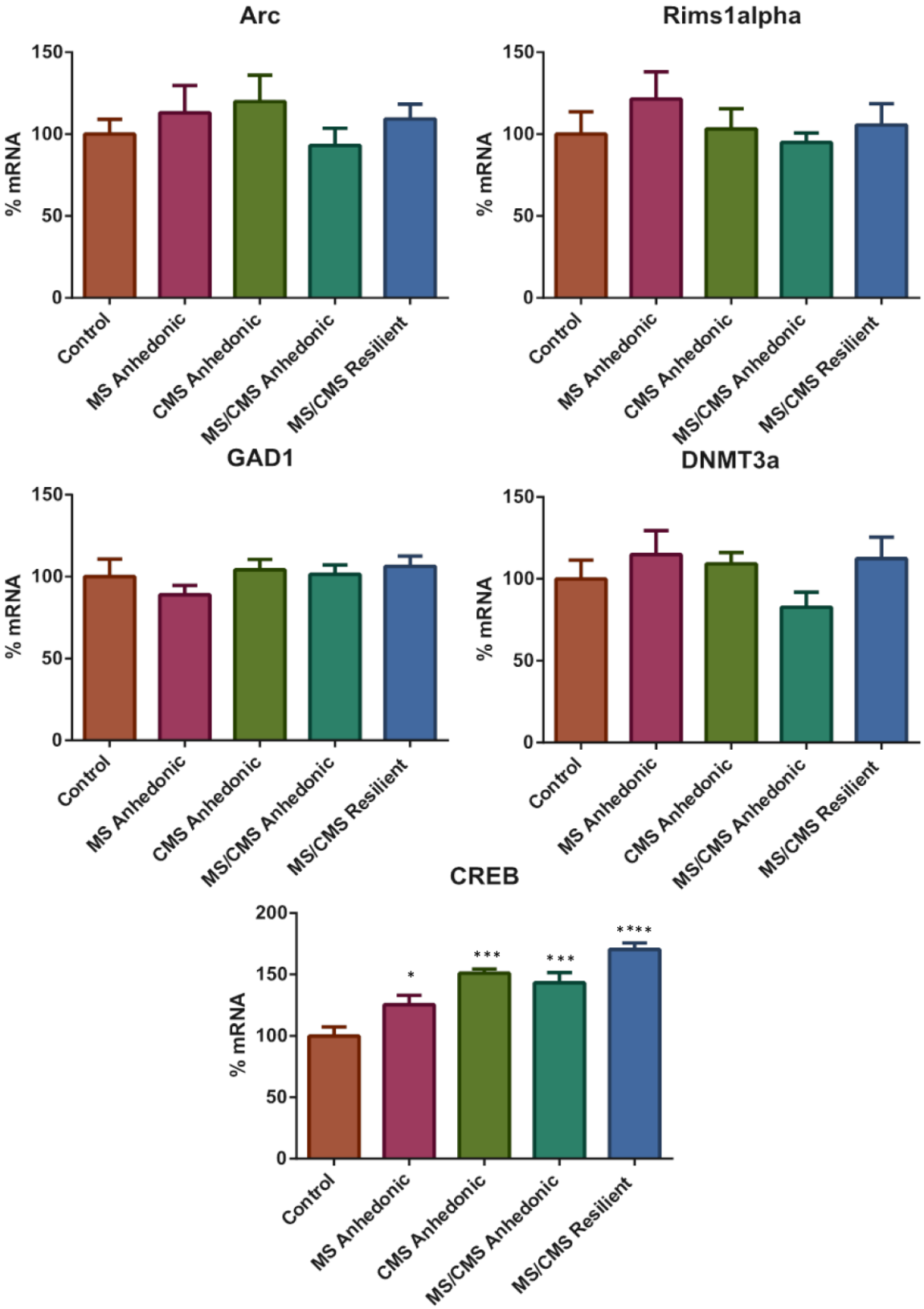


Figure 11 Expression of Arc, Rims, GAD1, DNMT3a, and CREB. n=6 for all groups except the CMS anhedonic where n=5. Data is presented as percentage of mean of the control group ±SE. The control group is set to 100 %. Significance levels shown on the figure are depicted as significant different from the control group. * represents P ≤ 0.05, *** P ≤ 0.001, and **** P ≤ 0.0001.

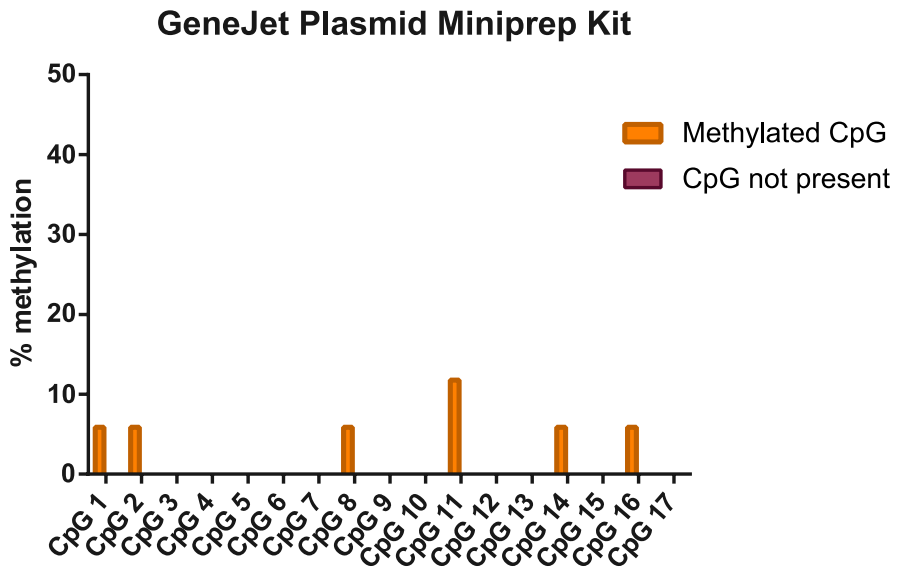
4.2 Sodium Bisulfite Mapping

The glucocorticoid receptor exon 1_7 promoter was chosen for a more thorough investigation of the methylational pattern of the promoter region. The promoter region covered contains 17 CpG sites, see Figure 12A. To ensure that the methylated cytosines was caused by methylation and not by incomplete conversion, the sequence conversion rates should be >97 %.

A

1757- cactt**cg**$_1$**cg**$_2$caactc**cg**$_3$cagttgg**cg**$_4$gg**cg**$_5$**cg**$_6$gaccacccctg**cg**$_7$gctctgc**cg**$_8$gctggctgtcaccct**cg**$_9$ggggctctggctgc**cg**$_{10}$
accca**cg**$_{11}$ggg**cg**$_{12}$ggctcc**cg**$_{13}$ag**cg**$_{14}$gttccaagcct**cg**$_{15}$gagctggg**cg**$_{16}$gggg**cg**$_{17}$ggagggag -1904

B



C

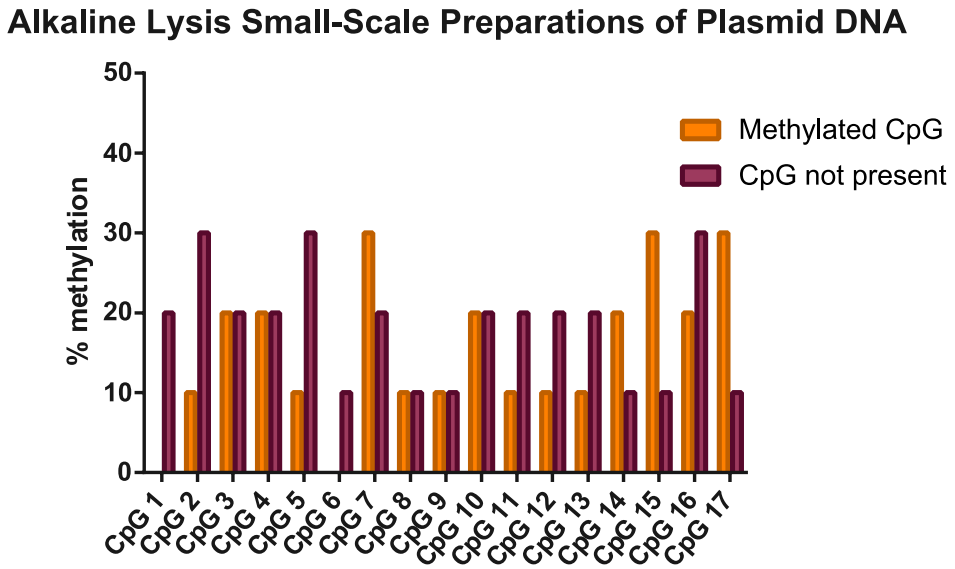


Figure 12 Methylation of the glucocorticoid receptor exon 1_7 promoter of animals from the MS anhedonic group. A) The genomic sequence of the glucocorticoid receptor exon 1_7 promoter, CpG sites is marked bold and numbered. B) Sequencing results obtained using the GeneJet™ Plasmid Miniprep kit for extraction of plasmids from bacteria, n=2. C) Sequencing results obtained using the Alkaline Lysis Small-Scale Preparations of Plasmid DNA for extraction of plasmids from bacteria, n=1.

Initiately, all plasmids were extracted from the bacteria using the GeneJet™ Plasmid Miniprep kit. The results obtained using the GeneJet™ Plasmid Miniprep kit showed average conversion rates of 99.86 % and 98 % for sequences of two animals examined by this method. However, due to limited resources, the Alkaline Lysis Small-Scale Preparations of Plasmid DNA method was used for further procedures. Unfortunately, when using the Alkaline Lysis Small-Scale Preparations of Plasmid DNA method the conversion rates was poorer, approximately 89 %. Sequences prepared with the latter method were of poor quality and did not match the original genomic sequence. Figure 12B shows the results obtained using the GeneJet™ Plasmid Miniprep kit for extraction of plasmids from bacteria, and Figure 12C shows the results obtained using the Alkaline Lysis Small-Scale Preparations of Plasmid DNA method. Note that the two Figures 12A and 12B are based on two and one animals respectively, and one animal is equivalent to 10 clones. When comparing the two methods, sequencing of the bisulfite treated DNA prepared using the Alkaline Lysis Small-Scale Preparations of Plasmid DNA method was more difficult compared to the other method. A lot of CpG sites were missing and the total methylational status turned out higher compared to sequences of bisulfite treated DNA prepared using GeneJet™ Plasmid Miniprep kit, even though animals were all from the MS anhedonic group, see Figure 12B and C. This could indicate that the Alkaline Lysis Small-Scale Preparations of Plasmid DNA method might not be suitable for preparing samples for sequencing.

4.3 Native Chromatin Immunoprecipitation

Through this study an optimization of a NChIP protocol was initiated. Native ChIP offers the opportunity to identify histone modifications associated with e.g. gene promoters. Application of NChIP in this study could potentially be used to describe the altered gene expressions found through the expression analysis. It is essential to maintain the linkage of histones to the DNA intact throughout the process. Therefore, the fractionation approach is crucial as the histones are not chemically cross-linked to the DNA. In this study fractionation of sample chromatin was done through sonication or through enzymatic digestion. First fractionation through sonication was tried as this method is faster and easier than enzymatic digestion. Initially, the Ultrasonic Processor was set to shear the chromatin at 10 % output power. However, this did not seem to shear the chromatin enough, the dark smear on Figure 13A is the fractionized chromatin. When shearing chromatin for NChIP the sheared fraction sizes should be approximately one to five nucleosomes in length, which is approximately 150-750 bp. The output power

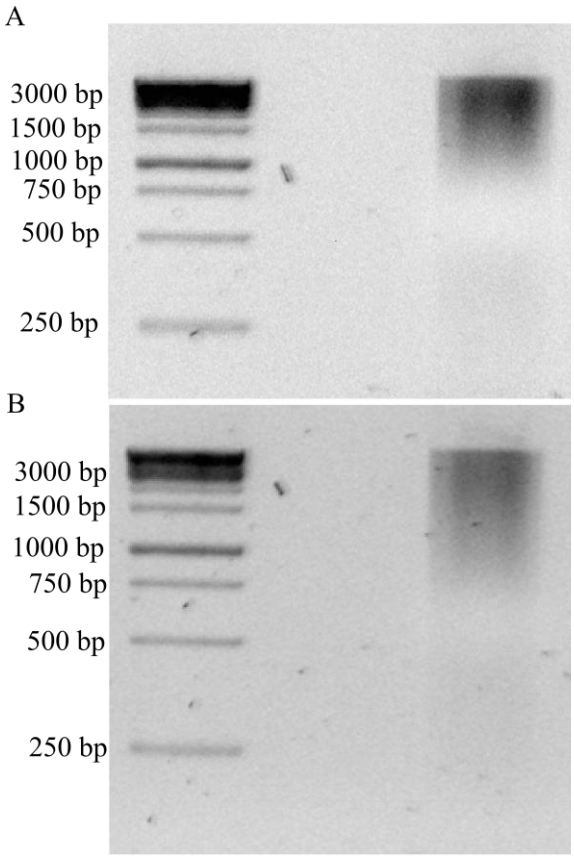


Figure 13 Sonication of sample DNA. A First attempt to fragmentize DNA using sonication, the output power was set to 10 %. B Second attempt to fragmentize DNA, here the output power was increased to 20 %. The ladder used was in both cases the GeneRuler 1 kb DNA ladder.

was therefore increased to 20 % in order to decrease fraction size. Unfortunately, this did not seem to improve the fraction size much, see Figure 13B. As we were afraid to disrupt the histone-DNA interactions by increasing output power even more due to the high amount of hydrodynamic shear forces created. Therefore, it was decided to try fractionation by enzymatic digestion. Digestion of chromatin with MNases is usually the method of choice when performing NChIP. First digestion was performed using 33 units for either 3 or 4 min., see Figure 14A. However, the chromatin seemed to accumulate in the P-fraction and to be caught in the well of the gel. Therefore, digestion for 4 min. using 75 units MNases was tried. However, this did not seem to solve the problem as the fractionized chromatin still accumulated in the P-fraction and seemed to be stuck in the well, se Figure 14B. Lastly, enzymatic digestion was increased to 10 min. using 75 units. This seemed to improve the assay slightly, however the chromatin only appeared in the P-fraction and it seemed as though most of the chromatin was still caught in the well, see Figure 14B.

The optimization of a NChIP protocol is far from done and more work is needed to optimize the fragmentation procedure. On behalf of these few attempts, shearing by sonication cannot be excluded as a possible approach to fractionation. Furthermore, the enzymatic digestion does not work properly and still needs optimization. Unfortunately, due to lack of time, the optimization was all we accomplished.

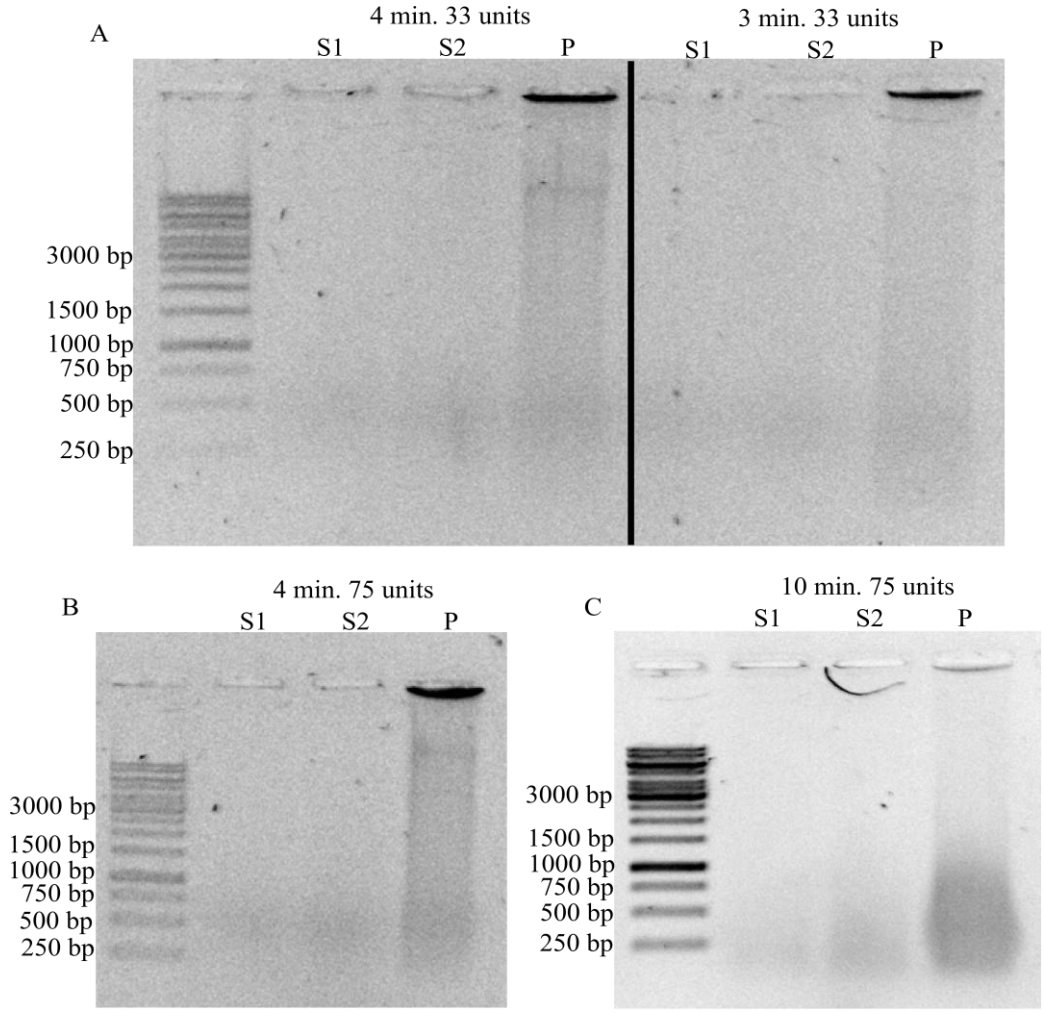


Figure 14 Enzymatic digestion of sample DNA. A) The first two attempts to cut the DNA using MNases, in both cases 33 units were used and the fragmentation time was 4 min. or 3 min. B) Second attempt to fragmentize DNA, the amounts of units was increased to 75. C) Third attempt, the amount of units was 75 and the fragmentation time was increased to 10 min. The ladder used was in all three cases the GeneRuler 1 kb DNA ladder.

5. Discussion

5.1 The heterogeneity of depression

To date one of the main obstacles in the search for a more comprehensive understanding of the pathology of depression is the fact that our limited knowledge renders it difficult to module a fully reliable animal model of depression. The complex interplay of the multifactorial pathophysiology of depression covering cognitive as well as homeostatic abnormalities hampers the establishment of an animal model mirroring the phenotypic and genotypic responses of depression in humans. Despite these obstacles several animal models of depression have been established. These models often capitalize on the evidence that adverse events often results in novel or recurrent depressive episodes. (Berton et al. 2012) Not only choosing the right animal model of depression is challenging. Choosing the right structure in the brain to examine is also obscured by the fact that not only one brain area is primarily related to depression. However, the hippocampal structure is one of the most widely investigated areas in regards to depression, though many questions are left unanswered (Nestler et al. 2002)

5.1.1 Animal Models of Depression

Animal models of diseases are widely used in order to study the pathophysiology and aetiology of a disease. However, some diseases render it difficult to establish an animal model by which the modelled symptoms are consistent with those of a humans. (Berton et al. 2012) The validity of the current animal models of depression is an impediment in the research of this complex disease. However, only animal models can help us understand the pathophysiology of depression and as we need more detailed knowledge about the pathophysiology of depression to module a good animal model, it is a vicious circle. (Berton et al. 2012)(Dzirasa and Covington, 2012)

Animal models can be judged along three different domains; face validity, predictive validity, and construct validity. Face validity is a daunting task when establishing a valid model of depression, as face validity refers to an animal models' ability to recapitulates important anatomical, biochemical, neurophysiological, or behavioural features of a human disease. Especially the anatomical, biochemical, and neurophysiological features of human depression remains a conundrum to be solved. Moreover, the behavioural alterations of depression seem different from patient to patient, and appears to include many distinct behavioural manifestations. (Dzirasa and Covington, 2012) The predictive validity on the other hand is an assessment of the animal models' ability to respond to a treatment, which should correspond to the effect obtained by the same treatment in humans. The third domain is the construct validity, which refers to the integration of relevant and observable characteristics associated with both the onset and the progression of a disease into the original design of the experimental animal model. (Dzirasa and Covington, 2012) Due to the poor understanding of the pathophysiology of depression, the current animal models of depression rely on known risk factors or valid aetiological alterations of the disease, which can be experimentally manipulated, resulting in traceable changes in behaviour and physiology. Hence, in a good animal model of depression the experimental animals' response pattern should resemble the human symptomatology of the disease in a reasonable manner. Moreover, the response pattern among the experimental animals should be similar to that of humans, in relation to the number of animals who succumb to the stressors and appear anhedonic in case of depressive models. (Nestler et al. 2002)

Despite several obstacles in regards to establishing a valid animal model of depression, the current models have enabled researchers to formulate several novel hypotheses by which depression might occur and how

antidepressants might work. (Nestler et al. 2002) Some of the more commonly used tests for modelling depressive behaviour are the forced swim test, tail suspension test, sucrose preference test, or the CMS model. (Dzirasa and Covington, 2012) The CMS model will be elaborated in the following.

The Chronic Mild Stress Model

One of the most frequently used animal models of depression is the CMS model. This model utilizes the well documented fact that exposure to mild stressors may result in development of depression. Through this model the animals categorized as depressed display anhedonia, one of the main symptoms of depression. Evidently not all animals are susceptible towards stress induced anhedonia, some animals does not succumb to the stressors and appear resilient in their hedonic profile. (Willner et al. 1992)(Henningsen et al. 2009) The frequency of anhedonic animals following execution of the CMS protocol is one of the main advocates when assessing if this model resembles depression in humans. Anhedonia is a major behavioural feature of human depressive episodes, which ensures some degree of face validity to this model. More importantly does the anhedonic behaviour of the animals persist beyond the CMS protocol timeframe. (Willner et al. 1992) As emphasized previously, achieving face validity is hampered by the lack of a more thorough knowledge of the pathophysiology of depression. (Dzirasa and Covington, 2012) The hedonic segregation of experimental animals included in a CMS paradigm is assessed by means of the animals' consumption of or preference for a sucrose solution. A decrease in sucrose consumption or preference is believed to be associated with anhedonia and further to reflect a decreased sensitivity to reward. (Henningsen et al. 2009) Another important aspect of the CMS paradigm is that the anhedonic animals can be relieved by administration of antidepressants. This is also imperative when considering that this animal model should resemble depression in humans. Furthermore, does the timeframe of recovery correspond to that of the clinical circumstances. Administration of antidepressants has shown to cause a return to baseline responsiveness to reward, which have been assessed by e.g. sucrose intake and place conditioning. (Willner et al. 1992) Moreover, it has been discovered that administration of non-antidepressants like anxiolytics does not reverse CMS induced anhedonia. This further supports the predictive validity of the CMS paradigm. (Dzirasa and Covington, 2012)(Willner et al. 1992)

5.2 The Role of Maternal Separation and Chronic Mild Stress on Gene Expression

As stated previously, the CMS model of depression is one of the most widely used animal models of depression. (Willner et al. 1992) New to the research area of depression is the MS model, which is thought to induce same phenotypic response as the CMS model. However, it remains uncertain whether the genotypic response of the two animal models are alike. In this section the results of the expression analysis will be discussed, and the two models will be compared.

5.2.1 Subunits of the AP-1 Complex

Through this study it was seen that both MS and CMS had an effect on the subunits of the AP-1 complex. The AP-1 complex consists of dimers of the Fos and/or the Jun protein families, thus the AP-1 complex refers to Jun:Jun and Jun:Fos/Fra dimers. Proteins of the Fos and Jun families are suspected to regulate the duration and stabilization of LTP, rather than the magnitude of LTP. (Herdegen and Leah, 1998) As LTP is an important process in learning and memory, which are altered during depressive episodes, the expression of these subunits might be implicated in mediating depressive behaviour. In this study all subunits was found

to be under the influence of MS and CMS, and of MS/CMS in combination. Interestingly three subunits shared the same expression pattern, these were: c-Fos, FosB, and JunB. It was seen that the CMS anhedonic rats had an evidently higher activity of c-Fos, FosB, and JunB compared to the control group. On the contrary the animals in the MS anhedonic group had a slightly lower expression of the three genes compared to the controls. Thus, the expression of all three subunits were higher in the CMS anhedonic group compared to the MS anhedonic group, which is very interesting as these animals shared the same phenotypic response. The last subunit of the AP-1 complex did not follow this exact expression pattern. Through the analysis of c-Jun it was seen that the CMS anhedonic group had a significantly higher expression of c-Jun compared to both the control group and the MS anhedonic group, this was similar to the other three subunits. However, the control group and the MS anhedonic group had more or less equal amounts of c-Jun expression. This indicates that though sharing the same phenotypic response, the animals of the MS anhedonic and CMS anhedonic do not share the same genotypic response. Hence, the underlying genetic mechanisms of the anhedonic behaviour induced by CMS appears to be different to those induced by MS as the AP-1 subunits are more widely expressed in the CMS anhedonic animals compared to the MS anhedonic animals. The expression of c-Fos, FosB, and JunB in the MS anhedonic group was moreover lower than the expression of those genes in the control group, thus emphasizing that the two groups do not share the same underlying genetic changes mediating the anhedonic phenotype.

The gene expression in the two experimental groups which have been exposed to both MS and CMS also differed among the AP-1 subunits. The expression of c-Fos, FosB, and JunB in the MS/CMS resilient group was higher than the expression in the MS/CMS anhedonic group. Opposing, the expression of c-Jun was lower in the MS/CMS resilient group compared to the MS/CMS anhedonic group. Unfortunately, little is known about the functions ascribed to the distinct subunits of the AP-1 complex, and the effects of these observed differences remain therefore to be further investigated. Furthermore, the functions of the subunits also depend on the counterpart of the dimer complex, thus making it even more complex to investigate. Nevertheless it has been found that the subunits are all implicated in regulation of the duration and stabilization of LTP, as the expression of the subunits correlates with the duration of LTP. (Herdegen and Leah, 1998) It is moreover very interesting that the combination of MS and CMS seems to reduce the expression of subunits c-Fos, FosB, and JunB compared to solely CMS. Opposing, in the expression analysis of c-Jun, exposure to MS alone did not have the same reducing effect on gene expression. The expression of c-Jun in the MS/CMS anhedonic was higher than the expression in the MS and CMS groups and the expression in the MS/CMS resilient group was somewhat the same as in both the MS and CMS anhedonic groups.

It seems that the MS and CMS paradigms have the same effect on expression of c-Fos, FosB, and JunB, but a somewhat different effect on the c-Jun expression. This might be attributed to the different ways of inducing expression of the subunits. Expression of the subunits is in general induced by various cytokines and growth factors such as Platelet-Derived Growth Factor (PDGF) and Epidermal Growth Factor (EGF). However, c-Jun is also greatly induced by DNA damaging agents like UV and Hydrogen Peroxide and moreover by the DNA cross-linker Mitomycin. (Herdegen and Leah, 1998) UV is proposed to be a more potent inducer of c-Jun compared to cytokines and growth factors. c-Jun is furthermore the only subunit capable of inducing cis-activation, as c-Jun:c-Jun dimers are able to bind to c-Jun promoters, creating a long lasting effect on the gene expression (Herdegen and Leah, 1998) The ability of these DNA-damaging agents to induce c-Jun expression and the cis-activating mechanism of c-Jun dimers may explain the deviating

expression pattern of c-Jun compared to the other subunits. Nevertheless, as the AP-1 complex is a transcription factor. Since little is known about the exact functions of the different subunits, and moreover of the functions of the different dimers, more research is needed in order to conclude how these genetic changes is anchored in the anhedonic phenotypic response of the MS and CMS models.

5.2.2 Down-stream Targets of the AP-1 Complex

Two down-stream targets of the AP-1 complex were included in this study to investigate whether the expression patterns of c-Fos, FosB, and JunB were propagated to these targets. The neuropeptides Gal and NPY are both down-stream targets of the AP-1 complex, and their functionally profile is closely related to depressive behaviour. It has been found that Gal is implicated in regulation of the serotonerigc system, across which many antidepressants exerts their action. The antidepressants categorised as monoamine oxidase inhibitors aim to increase the amount of 5-HT at the synapses. (Nestler et al 2002)(Ögren et al. 1998) Gal has been proven to inhibit 5-HT release in the hippocampus. Interestingly, in this study the expression of Gal was found to be decreased in the MS anhedonic group compared to the control group, which also accounts for the expression of c-Fos, FosB, and JunB. Suggesting that the expression of Gal is related to these AP-1 subunits in the MS anhedonic animals. The expression of Gal was moreover reduced in the MS anhedonic group compared to the CMS anhedonic group, which correlates with the hedonic phenotype of the animals, as the CMS experimental animals were more anhedonic than the MS animals (data not shown). This is in agreement with the literature, as it is found that an increased Gal expression decreases the availability of 5-HT at the synapses in the hippocampus, thus leaving the animals anhedonic. (Ögren et al. 1998) Opposing, the expression of Gal in the control group exceeds the expression observed in the MS anhedonic group and was somewhat the same as the expression in the CMS anhedonic group.

The expression analysis of Gal additionally revealed a reduced expression in the MS/CMS anhedonic group compared to the MS/CMS resilient group, which also was seen for c-Fos, FosB, and JunB. Opposing to the expression of these three subunits, the Gal expression was higher in the MS/CMS anhedonic and resilient groups compared to all other experimental animal groups. Interestingly, the same accounted for the expression of NPY. The expression of NPY was slightly higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group, and the expression in these two groups was higher compared to the control, MS anhedonic, and CMS anhedonic groups. This seems contradicting as these two neuropeptides in theory holds distinct functions. NPY on one hand positively regulate the HPA-axis serving to restore normal stress responses and thereby contributing to non-depressive behaviour, while Gal as described above decreases the quantity of 5-HT at the synapses in hippocampus thus attributing to depressive behaviour. (Cohen et al. 2012)(Ögren et al. 1998) These complex results need more clarification. It remains uncertain how two functionally distinct genes can share somewhat similar expression patterns. The results obtained could be caused by even more complicated genetic interactions underpinning the complex pathophysiology of depression. Nevertheless, one should keep in mind that the Gal primers used in this study may not reflect the actual expression of Gal, as the efficienciey was very high, see Table 1.

In the analysis of the NPY gene expression it was furthermore found that NPY was more widely expressed in the MS anhedonic group compared to the control group. Further, the expression was higher in the CMS anhedonic group compared to both the MS anhedonic group and the control group. This contradicts the findings of a previous study where NPY was proposed to be reduced in maternally separated rats. It should be noted that this decreased expression was found in the dorsal hippocampus. (Jimenez-Vasquez et al. 2001) Through our study the increased NPY expression in the MS anhedonic group and the CMS anhedonic

group compared to the control group should in theory result in a positively regulated stress response, which would decrease depressive behaviour. However, the animals of the MS and CMS groups are anhedonic in their phenotype. The expression was even further increased in the MS/CMS anhedonic and resilient groups compared to the three other animal groups. This increment would also result in a normally regulated stress response. On the other hand, the expression of NPY was very high in these two experimental groups compared to the controls, which could be speculated to contribute to the anhedonic phenotype of the MS/CMS anhedonic group as over-expression of NPY can result in damaging events. (Redrobe et al. 2002) However, this is not in agreement with the high expression seen in the MS/CMS resilient group in relation to their hedonic state.

5.2.3 Glucocorticoid Receptor

The expression of the GR was included in this study to investigate the influence of both MS and CMS on GR gene expression. GR is a major contributor to regulation of the HPA-axis, which in turn regulates the stress response. Thus alterations of the GR leads to an altered feedback mechanism resulting in a hyperactive HPA-axis, which might progress into depression. (Weaver, 2009) In the gene expression analysis of this thesis the total amount of GR and the fraction of GR containing the exon 1_7 were investigated. The analysis of total GR did not reveal any significant changed expression among the five experimental groups. This is not in agreement with the findings of other studies. Previously it has been found that maternal care decreases the expression of GR. In a study by Liu et al 1997 they examined the effect of maternal care on HPA-axis sensitivity. They demonstrated that pups of high nursing mothers had a decreased HPA response to stress in adulthood by means of lower plasma ACTH and corticosterone responses to restraint stress. They further found that the expression of GR was increased in the pups reared by high maternal care mothers compared to those reared by low maternal care mothers. The GR mRNA expression was in fact significantly correlated with the frequency of maternal licking and grooming in both CA1, CA3, and the DG of hippocampus. The pups reared by maternal caring mothers thus displayed an increased HPA response sensitivity and increased expression of GR, resulting in a dampened HPA response to stress. Whilst the pups of low maternal care mothers showed a decreased expression of GR in the hippocampus, leading to HPA-axis hyperactivity and thereby increased stress responses. (Liu et al. 1997) However, through this study neither MS nor CMS seems to have an influence on total GR expression. The decrement in GR caused by low maternal care seen by Liu et al. was observed in adult rats, which was not the same response observed in our adult MS deprived rats. Liu et al. dissected the CA1, CA3, and DG areas of hippocampus, which could be the explanation for the discrepancy between the two studies. The expression patterns of total GR might be different when dissecting the CA1, CA3, and DG, which prolong into the dorsal hippocampus, opposed to using only the ventral hippocampus as was done in this thesis.

In line with the altered GR expression, other studies have previously shown that the GR exon 1_7 promoter is associated with increased methylation in pups exposed to decreased maternal care and/or stressful events. In one study it was found that the promoter region of the GR exon 1_7 was heavier methylated in adult offspring of low maternal care dams compared to offspring of high maternal care dams. (Henningsen et al. 2012) Hence, increased methylational status of the promoter region would inhibit transcription resulting in limited expression of the gene. Moreover, Weaver et al. found the GR exon 1_7 promoter region to be heavily methylated in pups of low maternal care mothers compared to pups reared by high maternally caring dams. (Weaver et al. 2004) Opposing, it was in our study found that the expression of GR exon 1_7 was higher in the MS anhedonic group compared to the control group and the CMS anhedonic group. This

suggests that the animals of the MS anhedonic group had a more sensitive HPA response and thereby decreased stress response compared to the animals of the CMS anhedonic group. This is in agreement with the general hedonic state of the animals in the two groups. As previously mentioned, the animals in the CMS group were more anhedonic compared to those in the MS anhedonic group. Furthermore, it was found that the expression of GR exon 1_7 was slightly higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group, which also is in agreement with the hedonic phenotype of the animals. However, when comparing our results of the GR exon 1_7 expression with the previous findings of Henningsen et al. and Weaver et al. they seem contradicting. (Henningsen et al. 2012)(Weaver et al. 2004) Based on the heavy methylational status of the GR exon 1_7 promoter region induced by maternal care, one might expect the expression of GR exon 1_7 to be lesser in the three anhedonic groups compared to the control group. However, as previously described, the epigenome is thought to be dynamic and methylations can be reversed. This could provide an explanation for these observations. Thus creating the opportunity that GR exon 1_7 initiately might be heavily methylated, where after the methylations are reversed and transcription of GR is initiated as a response to a diminished GR density.

5.2.4 Plasticity Related Genes

The plasticity related genes Arc and Rims1α were included in this thesis to examine the effect of MS and CMS on these two genes. Both genes are critically involved in plasticity related changes. Arc has been associated with consolidation of both LTP and LTD thus making Arc essential for regulation of synaptic homeostasis. Rims1α is related to synaptic long term plasticity and consolidation, which are processes essential for maintenance of cognitive behaviour. (Kaeser et al. 2008)(Kaeser and Südhof, 2005)(Coppens et al. 2011)(Bramham et al. 2008)

In this study, the expression of Arc was found to be increased in the MS anhedonic group compared to the control group, and further increased in the CMS anhedonic group compared to the two other groups. As the processes of synaptic homeostasis might be altered with increased anhedonic phenotype, the high expression of Arc seen in the CMS anhedonic group could be an attempt to restore synaptic homeostasis. This correlates with the slightly minor anhedonic phenotype described among the MS anhedonic animals and the slightly decreased expression of Arc in this group compared to the CMS group. In another study, Arc was found to be increased in the hippocampus following social defeat in adolescent rats, but not in adult rats. The increased expression of Arc following social defeat was even present 4 hours subsequent to the stressful event in the young rats. (Coppens et al. 2011) Thus, indicating that induction of Arc expression might be important in the early life adverse events. Intriguingly, the combination of MS and CMS diminishes the expression of Arc among the anhedonic animals. This indicates that the expression is not as simple as just adding the effect observed in the animals of the MS and CMS groups. Further, it was seen that Arc expression was higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group. This suggests that the role of Arc might be different in the resilient animals compared to the anhedonic animals.

The plasticity related gene Rims1α was also increased in the MS anhedonic group compared to the control group. Interestingly, the expression of Rims1α was lower in the CMS anhedonic group compared to the MS anhedonic group, which might indicate that the expression of Rims1α is not related to the degree of anhedonia observed in the animals. Moreover, it was found that the expression of Rims1α was increased in the MS/CMS resilient animals compared to the MS/CMS anhedonic animals. This was also the case for the expression of Arc, again emphasizing that the genetic alterations might be different in the resilient animals

compared to the anhedonic animals. In regards to the Rims1α expression it seems as if the combination of MS and CMS decreased the expression in the MS/CMS anhedonic animals. When comparing Arc and Rims1α the genetic alteration related to plasticity is not similar in the MS model compared to the CMS model. However, the expression of both Arc and Rims1α is increased in the resilient MS/CMS animals compared to the MS/CMS anhedonic animals.

5.2.5 cAMP Response Element-Binding protein

The involvement of transcription factor CREB in depressive behaviour is abundant. CREB is involved in regulation of transcription of some Jun:Fos dimers, Arc, and GR, all relevant to the aetiology of depression. (Herdegen and Leah, 1998)(Lam et al. 2009)(Weaver et al. 2001) CREB has furthermore been found to be an important regulator of consolidation of learning and memory. (Nestler et al. 2002) Through this study it was found that the expression of CREB was increased in the MS anhedonic group compared to the control group, and further increased in the CMS anhedonic group compared to the two other groups. This increment was interestingly also found in the Arc expression analysis, and Arc is a down-stream target of CREB. Furthermore, the expression of CREB was also higher in the MS/CMS resilient group compared to the MS/CMS anhedonic group. This was also the case for the expression of Arc. The expression patterns for the two genes show similar tendencies, indicating that the changed Arc expression might be mediated by CREB. Similar to Arc, the anhedonic phenotype seems to correlate positively with the expression of CREB. The tendency of increased CREB expression found in the MS/CMS resilient group compared to the MS/CMS anhedonic group was also present in the analysis of GR exon 1_7, and moreover for the three AP-1 subunits c-Fos, FosB, and JunB. This suggests that transcription factor CREB might play an important role in the MS/CMS anhedonic and MS/CMS resilient genotypic responses.

5.2.6 DNA Methyltransferase-3a

Over the last decades some of the genetic alterations found in depressive behaviour mediated by maternal handling have been postulated to arise from epigenetic modifications. One major contributor to these modifications is the DNMT3a, which was included in this expression analysis. (Weaver, 2009) Through the analysis of DNMT3a the mRNA level was found to be increased in the MS anhedonic group compared to both the controls and the CMS anhedonic group. This corresponds to the involvement of DNMT3a in *de novo* methylations. (Weaver, 2009) Other studies have observed that a decrease in maternal care and exposure to stressors induce increased methylational activity at promoter regions, thus the expression of DNMT3a and DNMT3b would be increased with increased methylations. (Zhang et al. 2010)(Henningsen et al. 2012)(Weaver et al. 2004) Our results points towards an increased methylational activity in the MS anhedonic group compared to the CMS anhedonic group. Opposing, the DNMT3a expression was lower in the MS/CMS anhedonic group compared to all other groups. This suggests that the genetic alterations of this group compared to the MS and CMS anhedonic groups, might be more complex. It is worth to note that methylations of DNA are dynamic processes, which can be reversed. The expression of DNMT3a was in addition found to be associated with accessibility of the Arc gene. (Oliveira et al. 2012) The direct mechanism behind this correlation is yet to be discovered. However, there is some similarities between the expression patterns of the two genes, thus indicating that DNMT3a could be interfering with Arc expression.

5.2.7 Glutamic Acid Decarboxylase-1

The last gene included in this study was the GAD1 gene. GAD1 is involved in the synthesis of GABA from Glu, this process is essential to ensure homeostasis among the Glutamatergic and GABAergic System.

(Zhang et al. 2010) However, the expression analysis revealed that only MS seems to have a slightly reducing effect on GAD1 expression. The expression was more or less the same in all other experimental animal groups. This is somewhat in agreement with previous findings. In a study by Zhang et al 2010 it was found that decreased maternal care decreased the expression of GAD1 while an increased maternal care similarly increased GAD1 expression. (Zhang et al. 2010) Through this study the pups included in the MS protocol also displayed a slightly decreased GAD1 expression. Opposite, CMS do not seem to have an effect on the expression of GAD1, again emphasizing that the genotypic response of the MS anhedonic rats may not be comparable with that of the CMS anhedonic rats.

6. Conclusion

Through this thesis it was found that both MS and CMS had an effect on the genes analysed through the gene expression analysis. In some genes the expression correlated positively with the hedonic response of the experimental animals when comparing to the control animals. However, other expression analyses revealed that the induction of MS or CMS resulted in distinct mRNA levels. This could indicate that the genetic alterations mediated by induction of MS and CMS is somewhat different. Hence even though the animals of the MS and CMS experimental groups share an anhedonic phenotype, the underlying genetic changes mediating this phenotype might not be similar. Furthermore, when comparing the two animal groups exposed to both MS and CMS, it is very interesting that the gene expression is higher for all genes except c-Jun. This also indicates that the genotypic response of these groups are different. Though the animals of the MS/CMS resilient group phenotypically are similar to the control group, the gene expression levels are surprisingly not similar to that of the control group.

It was also found that the expression pattern of AP-1 complex subunits c-Fos, FosB and JunB were similar in all experimental groups. Which is an interesting observation as these three subunits are all regulated by the same cytokines and growth factors. Moreover, it was found that transcription factor CREB and its downstream target Arc also shared expression patterns in all experimental groups. Thus indicating that CREB is involved in regulation of Arc in relation to MS and CMS.

7. References

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