Early Life Stress-induced Histone Acetylations Correlate with Activation of the Synaptic Plasticity Genes Arc and Egr1 in the Mouse Brain

Dissertation

zur Erlangung des akademischen Grades

doctor rerum naturalium
(Dr.rer.nat)

genehmigt durch die Fakultät für Naturwissenschaften

der Otto-von-Guericke-Universität Magdeburg

von MSc. Lan Xie

geb. am 02.11.1980 in Hunan, Volksrepublik China

Gutachter: PD Dr. Jörg Bock

Prof. Dr. PhD. Richard Nass

eingereicht am: 06.03.2013

verteidigt am: 18.07.2013

Content

Histon-Acetylierung nach frühkindlichem Stress korreliert mit Aktivierung der plastizitätsrelevanten Gene Arc und Egr1 im Gehirn der Maus

Vorgelegt von Msc. Lan Xie

Zusamenfassung

Stress, besonders wenn er im Kindesalter erlebt wird, hat lang anhaltende Konsequenzen für den Organismus, sowohl auf Verhaltensebene, als auch auf molekularer Ebene, die die Entwicklung des Gehirns betreffen. In Abhängigkeit von Qualität, Dauer und Zeitpunkt des Stressors, kann dies das Risiko für psychische Krankheiten erhöhen oder aber zu Resilienz und erfolgreicher Stressbewältigung im Erwachsenenalter führen. In der vorliegenden Arbeit wurde der Einfluss von Histon-Acetylierung auf die Expression plastizitätsrelevanter Gene, die an der neuronalen und synaptischen Reifung emotionsrelevanter Gehirnregionen beteiligt sind, untersucht.

Mutterseparation (MS) von Postnataltag 14 – 16 diente als früher Stressor, dessen Einfluss auf zwei Schlüsselgene der synaptischen Plastizität, Arc und Egr1, untersucht wurde. In Genexpressionsanalysen unmittelbar nach dem letzten Stressor wurden erhöhte mRNA-Levels von Arc und Egr1 im Hippokampus jedoch nicht im präfrontalen Kortex (PFC) von gestressten Mäusen im Vergleich zu ungestressten Kontrolltieren gemessen. Weiters konnte durch Immunoblotting ein signifikanter Anstieg der Acetylierung der Histone H3 und H4 im Hippokampus Stress-exponierter Tiere nachgewiesen werden., während im PFC lediglich eine Erhöhung der Acetylierung von Histon H3 auftrat. Mit Hilfe der nativen Chromatin Immunopräzipitation-quantitativen Polymerase Kettenreaktion (ChIP-qPCR) konnte nachgewiesen werden, dass die erhöhte H4-Acetylierung im Hippokampus gestresster Mäuse mit den Promoterregionen von Arc und Egr1 assoziiert ist. Dies deutet darauf hin, dass die beobachteten Histon-Modifikationen direkt mit der erhöhten Expression der beiden Gene in Zusammenhang stehen. Obwohl mittels Immunoblot nur eine allgemein erhöhte Histon H3 Acetylierung im PFC gemessen wurde, konnte mittels nChIP-qPCR eine spezifisch erhöhte Acetylierung von H3 und H4 an den Promotoren von Arc und Egr1 nachgewiesen werden.

Zu späteren Entwicklungszeitpunkten konnte zudem eine Zunahme der Komplexität der Dendriten von hippokampalen CA3-Pyramidenzellen nachgewiesen werden. Dies ging mit einer Reduktion des Passivverhaltens im Forced Swimming Test (FST) und einer Verbesserung räumlichen Lernens einher.

Zusammenfassend legen meine Ergebnisse den Schluss nahe, dass sich wiederholtes Erleben von relativ geringem Stress in der frühen Entwicklung positiv auf Hirnentwicklung und Verhalten auswirkt. Es wird spekuliert, dass die Stress-induzierte Transkription von Arc und Egr1 , reguliert durch Histonacetylierung , einer der ersten Schritte in neuronaler Plastizität und der Anpassung neuronaler Netwerke an eine Stress-induzierende Umwelt ist.

Early Life Stress-induced Histone Acetylations Correlate with Activation of the Synaptic Plasticity Genes Arc and Egr1 in the Mouse Brain

Submitted by Msc. Lan Xie

Abstract

Early-life stress programs the developing organism and influences the development of brain and behavior. Depending on the type of stressor and the duration and timing of stress-exposure this can be a critical factor for the development of behavioral dysfunctions and mental disorders but also for a positive adaptation regarding stress coping later in life. Here the hypothesis tested was that histone acetylations result in alterations of synaptic plasticity-related genes, which are critically involved in the neuronal and synaptic development of emotionally relevant brain circuits and behaviours.

Using repeated maternal separation (MS) from PND 14-16 as an early life stressor, two immediate early genes underlying experience-induced synaptic plasticity were analyzed, Arc and Egr1. Gene expression analysis immediately after the last stress exposure revealed elevated Arc and Egr1 mRNA in the hippocampus but not in the PFC of stressed animals compared to unstressed controls. Additionally, immunoblotting revealed an immediate significant increase in the levels of acetylated histone H3 and H4 in the hippocampus of stressed animals, whereas in the PFC only acetylated histone H3 was increased. Using native chromatin immunoprecipitation-quantitative polymerase chain reaction (n-ChIP-qPCR) it was found that the increased acetylation of H4 in the hippocampus of stressed mice was associated with the promoter regions of Arc and Egr1 genes, indicating that the observed histone modifications are directly correlated to the increased gene expression. In the PFC of stressed animals, increased acetylation of both histone H3 and H4 were observed specifically at the promoter regions of Arc and Egr1. Moreover, at later developmental time points, an increase of dendritic complexity and number of spines in CA3 pyramidal neurons was observed in the stressed animals, which was paralleled by a reduction of depressive-like behaviour in forced swimming test (FST) and an improvement of spatial learning.

We demonstrate for the first time that early life stress up-regulates histone acetylation levels around the promoter regions of the synaptic plasticity genes Arc and Egr1 using n-ChIP-qPCR, resulting in an increased expression of these genes in the hippocapus. It is tempting to speculate that these epigenetic mechanisms interfere with the establishment and "formatting" of hippocampal circuits and thereby induce long-lasting structural changes in hippocampal synaptic connectivity and behavioural changes.

1. Introduction

1.1 Early Life Stress

1.1.1 Human studies

A growing number of epidemiological studies have indicated that experience of stressful events during childhood exerts a profound and pervasive influence on emotional behaviour, and increases the risk of depression, anxiety disorders and substance abuse disorders (Heim and Nemeroff, 2001; Khoury et al., 2010; Keyes et al., 2011). Early life stress for children including maternal anxiety/depression, psychosocial and environmental stressors also increases the risk of immune system diseases (Vig et al., 2010; Kozyrskyj et al., 2011; Wright 2012). Based on the cited studies, it is clear that psychological disorders, which may result from early life stress can vary. What causes this diversity? Apart from the timing and the strength of stressful events, the genetic factor cannot be ignored. Meta-analysis on twin studies suggests that the heritability rate of major depression is between 31%-42% (Sullivan et al., 2000). There are many twin and adoption studies supporting the hypothesis that there is a high incidence of psychological diseases in the populations with genetic predispositions, which can be connected to stressful environmental factors (Dick, 2011; Nugent et al., 2011). Further, this correlation is more significant in individuals with a more transient and stressful environment in childhood. MAOA (monoamine oxidase-A), 5-HTT (serotonin transport) and DRD4 (dopamine receptor D4) are well-studied vulnerability genes correlated to stressful events. Children carrying either low-MAOA-activity allele, short allele of 5-HTTLPR (5-hydroxytryptamin-linked polymorphic region polymorphism) or 7-repeat DRD4 allele are more susceptible to stressful events and the development of disorders (Belsky et al., 2009).

In addition to the genetic variation, it is also important to address the question of how the environment influences biological processes and affects outcomes? An epidemiological study has reported that 13-year-old adolescents from mothers suffering from depression during the early postnatal periods show higher baseline glucocorticoid levels, which predicts a depressive symptomatology by 16 years of age (Halligan et al., 2007). A similar elevation of basal levels of glucocorticoids was also observed in adolescents who grew up in an environment of poverty (Evans and English, 2002). It has been revealed that stress-related neuropsychiatric disorders result from a dysfunction of the hypothalamic-pituitary-adrenal axis (HPA axis) (for a review, see Yehuda and Seckl, 2011). Under normal conditions, stress stimulates the release of corticotropin releasing factor (CRF) from the hypothalamus and causes the pituitary to release adrenocorticotropic hormone (ACTH), which transports the

signal to the adrenal gland and increase glucocorticoid levels. Glucocorticoids conduct the signals to their receptors in hippocampus and hypothalamus, followed by negative feedback loops and thus inhibition of the HPA axis activity. Under sustained stress, the HPA axis remains activated, which leads to a release of excessive amounts of glucocorticoids. This long-lasting reaction results in a down-regulation of glucocorticoid receptor (GR) expression and impairs the negative feedback of HPA axis (For a review, see Checkley 1996; Maccari and Morley-Fletcher 2007; Mizoguchi et al., 2008). GR signaling not only regulates the negative feedback of HPA-axis, but also regulates the biological actions in different tissues and eventually manifests various stress-related diseases.

1.1.2 Animal models of stress

To facilitate the research on human stress-related diseases, animal models for stress have been established in the labs instead. To mimic human stressful events in animal models, physical and emotional stimuli are the most commonly used approaches to induce stress in animals. Commonly used stressors are briefly described as follows and single or repeated stressful event applications are described as acute or chronic stresses respectively.

Restraint or immobilization: Restraint stress is induced by placing the animal in a tightly fitted plastic cylinder with one end closed for a certain period to immobilize the animal (Maccari et al., 2007; Saruta et al., 2010).

Inescapable foot shock: The animal is placed into one of two compartments that are equal in size, separated by a wall with a central open door. The electric current is applied to the stainless-steel grid floor. A certain series of inescapable shocks is delivered to the compartment that the animal is in (Pynoos et al., 1996; Philbert et al., 2012).

Forced swim: This procedure consists of inescapable exposure to a cylinder filled with water at room temperature. The duration of the session can be varied from 6 to 15 min (Porslot et al., 1977; MacQueen et al., 2003; Veenema et al., 2006; Lee et al., 2007; Pollak et al., 2010).

Social Isolation: Isolation procedure usually is conducted during the first week after post-weaning. Isolated rodents are housed individually in the same husbandry conditions, however they are not able to have social interaction with each other (Fone and Porkess 2008).

Social defeat: This stress contains both a physical and a psychological component. The experimental subject as an intruder is placed into the home cage of an aggressive resident for a certain period of time, allowing the intruder to be attacked by the resident (Koolhaas et al., 1997; Huhman, 2006).

Maternal Separation: Separate dams and pups in early life for several consecutive days, while 3-6 hours per day. Generally, this includes two paradigms: (i) The dams are transferred to new cages and keep the pups in homecages (Plotsky and Meaney, 1993; Zimmerberg and Sageser, 2011; Babygirija et al., 2012) (ii) The dams are remained in homecages, the pups are transferred to new cages and isolate individuals in cages (Pryce et al., 2003; Bock et al., 2005a; Gruss et al., 2008; Zimmerberg and Sageser, 2011).

Prenatal stress: Daily predictable or unpredictable stress, such as restraint, electric shoc, elevated platform and others, on rodent dams during the last week of pregnancy (Takahashi et al., 1992; Henry et al., 1994; Welberg et al., 2000; Murmu et al., 2006; Bock et al., 2011; Harris and Seckl 2011).

As observed in humans, stress elevates stress hormone levels and chronic stress leads to impairment of the HPA axis in animals (Weinstock 1997; Charmandari et al., 2005). Different behavioural tests are used to measure the stressed animals mood-related behaviours and cognitive functions (Cryan et al., 2002), for example, forced swimming, inescapable shocks and tail suspension are used to test helplessness (Porsolt et al., 1977; Geyer and Markou 1995; Lucki 1997; Mangiavacchi et al., 2001; Cryan et al., 2005; York et al., 2012), sucrose preference or intake tests assess anhedonia (Geyer and Markou 1995; York et al., 2012); Open field and elevated plus maze test for anxiety (York et al., 2012). Furthermore, fear conditioning, Morris water maze, novel object recognition and others are widely used to assess cognitive functions in animals (York et al., 2012). Using these behavioural, cellular and molecular tests or examinations, it has been well documented that early life stress alters animal behaviour and induces complex pathological characteristics similar to those in humans.

In the present study, maternal separation was employed as early life stressor. Therefore, a more detailed description of this widely used paradigm is given in the next chapter.

1.2 Maternal Separation Models

1.2.1 Behavioural and morphological consequences

Maternal separation (MS) or maternal deprivation is widely used to investigate the effects of early life stress in animal models. Up to today, there is no standardized paradigm. In most cases, acute 24 hours of MS, it is also called maternal deprivation, and 1-3 weeks of 1-6 hours of daily MS during the first 3 weeks of a newborn rodent are applied (Stanton et al., 1988; van Oers et al., 1998; Wigger and Neumann 1999; George et al., 2010; Schmidt et al., 2011; Tata 2012; Wei et al., 2012a). Evidence from these models shows that MS has effects on neuroendocrine function, emotional and cognitive behaviours, such as HPA axis function, depressive/anxiety-like behaviours. At the behavioural level, animals subjected to MS in early life display significantly increased anxiety-like behaviours reflected by reduced entries and spending less time in the center area in open field (Francis et al., 2002; Lambás-Señas et al., 2009), and spending less time in the open arms of elevated plus maze (Huot et al., 2001; Kalinichev et al., 2002; Asia et al., 2007). MS pups also show depressive-like behaviours, such as increased immobility time in forced swimming test (MacQueen et al., 2003; Veenema et al., 2006; Lee et al., 2007; Lambas-Senas et al., 2009), reduced sucrose intake (Huot et al., 2001; Aisa et al., 2007), and cognitive impairment in object recognition tasks in late life (Asia et al., 2007; Hulshof et al., 2011; Martisova et al., 2012). However, it is worth noting that MS stress is not the only factor considered in an MS model. Exploration of a novel environment during the separation and maternal care displayed after the reunion should also be considered. It has been well-documented that MS motivates the mother to spend significantly more time on the nest immediately after reunion and alters maternal behaviours upon reunion (Liu et al., 1997; Boccia and Pedersen 2001; Zimmerberg et al., 2003; Millstein and Holmes, 2007). And other studies using different mouse strains also show that an increase in maternal care after reunion potentially reduces the effects of separation itself (Millstein et al., 2006; Millstein and Holmes 2007). Maternal care has been demonstrated to reduce stress responsiveness via suppressing hypothalamic CRF in the hypothalamic PVN together with CRF receptor expression in the amygdala perhaps by enhancing hippocampal GR expression (Liu et al, 1997; For a review, see Walker, 2010; Aniko and Baram, 2011). However, it is also worth noting that maternal care after reunion does not certainly reduce depressive-like and/or anxiety-like behaviours in long-term (or chronic) stressed models. Three weeks of brief daily separation (40 min) in early life increase maternal care including licking, grooming, duration of nursing and time spent with pups, however, MS pups demonstrated anxiety-like behaviours (Wei et al., 2010).

In aspect of neuromorphology, most studies reported that stress represses synaptic structures (Radley et al., 2004; Pascual and Zamora-León 2007; Takatsuru et al., 2009; Monroy et al.,

2010; McEwen et al., 2012). However, some studies reported that chronic MS increases spine density and branch length in subregions of the mPFC (Muhammad and Kolb, 2011; Muhammad et al., 2012). Moreover, some studies have found that the morphological changes in dendrites under stressful conditions are time-dependent (Bock et al., 2005a; Gos et al., 2008; Monroy et al., 2010). Our previous studies distinguished the dendritical morphological consequences induced by MS in different time-windows (*i.e.* before/during/after stress hyporesponse period (SHRP) of the HPA axis) (Bock et al., 2005a; Gos et al., 2008). It has been observed that decreased spine density occurred only in layer II/III pyramidal neurons of the anterior cingulate cortex (ACd) only if MS is conducted before SHRP. No changes occur during SHRP and spine density is increased by MS after SHRP (Bock et al., 2005a). Thus, it is assumed that the dendritical structure changes as a result from MS stress, are sub-region/neuron-specific and time-frame dependent, which may be regulated by the HPA axis. Evidence shows that chronic stress in childhood desensitizes the HPA axis response to acute stress, as well as impairs the spatial memory and avoidance learning in adulthood (for a review, see Lupien et al., 2009; Tata 2012). Pharmacologically, corticosteroid administration exerts different effects in dendrite development and facilitates the growth of dendrites in 3-weeks-old rats, whereas it represses dendritic growth when the animal is 6-weeks-old (Oda and Huttenlocher, 1974). Thus, it is assumed that the morphological alterations might result from changes in the HPA axis. These findings suggest that molecular, cellular and/or behavioural consequences of early life stress in rodents are originally adjusted by stress hormones (Oda and Huttenlocher, 1974; Rosenfeld et al., 1992; Bock et al., 2005a; Tata 2012).

1.2.2 Adaptation

The consequences produced by stress are dependent on the type of stress, its duration and the age during the exposure. Stress does not always exert negative effects on brain development and behaviour. There is increasing evidence that stress may also induce adaptations for better coping with environmental, cognitive and emotional challenges later in life (For a review, see McEwen and Gianaros, 2010; Karatsoreos and McEwen, 2011). For instance, as it was shown in recent studies, MS stress or prenatal stress can exert positive effects on the development of emotional as well as cognitive competence (Sanders and Anticevic, 2007; Schäble et al., 2007; Abraham and Gruss, 2010; Schroeder et al., 2012). The question is how the brain copes with stressful events?

1.2.3 Stress response and the HPA axis

HPA axis is a complex component of neuroendocrine regulatory system (see above). It initiates the response to stress in many ways, such as influencing emotional and cognitive functions. In addition, evidence in humans and animals indicate that three major parts of the brain are involved in coping stressful events (figure 1.1, adapted from McEwen and Gianaros, 2010). These are the hippocampus, subregions of the prefrontal cortex (PFC) and the amygdala (McEwen and Gianaros, 2010). The network among these regions allows individuals to learn and decide whether stressful events are harmful or escapable/inescapable, and further induce behavioural habituation or dysfunction.

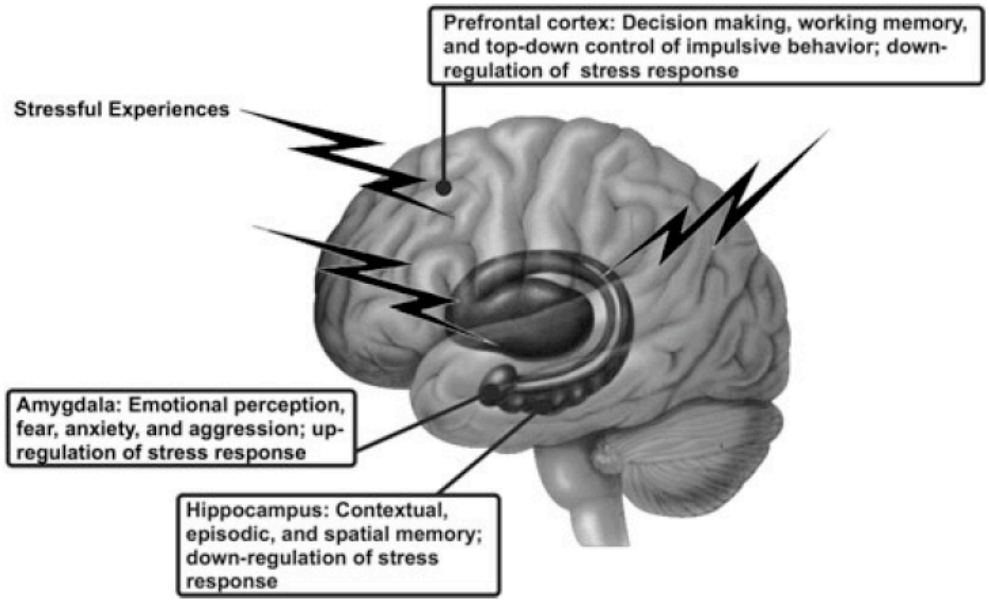


Figure 1.1: Schematic illustration of the location and key functions of limbic brain areas. (Adapted from McEwen and Gianaros, 2010)

The PFC is a neocortical region that is the headquarter of the projections to or from the sensory and the motor cortex, as well as subcortical structures including the hippocampus and the amgydala (For a review, see Miller and Cohen, 2001). Also, the medial PFC (mPFC) regulates HPA axis activity responding to stressful events (Diorio et al., 1993; Brake et al., 2000), and takes a crucial role in emotional and cognitive processing in association with the hippocampus (For a review, see Vertes, 2006).

In MS model, mother absence is an emotional stressor and stimulates alterations in the PFC of isolated pups, while the new environment may play a role of stressful context that relays the stimulation to the hippocampus. Therefore, the repeated MS allows pups to learn and to

remember declarative and spatial information, which may result in an increase in hippocampal plasticity for the adaptation to stress. On the other hand, stress hormone-regulated GR is widely expressed in the rodent brain and condensed in the hippocampus: hippocampal GR is assumed to restore homeostasis via feedback-regulation of the HPA axis (McEwen et al., 1986; De Kloet et al., 1987; Sousa et al., 1989; Jacobson and Sapolsky, 1991). The duration and the strength of stressors stimulate these brain regions to either regulate the homeostasis to adapt to stress or be impaired to negative feedback. Taken together with our previous findings (Bock et al., 2005a,b, 2011, 2012), the present study investigated in alterations in the PFC and the hippocampus .

1.3 Mechanisms underlying Stress

In the last century, many mechanisms underlying stress response were revealed, from early findings, such as theory of neurotransmitter and genetic theory to more recent theory of imaging and neuronal morphology. Furthermore, with development of molecular biology, possible epigenetic mechanisms are raised-up in neuroscience. This chapter introduces some well-studied mechanisms underlying stress in brief, and focus on rising aspects of dendritic remodeling and epigenetics.

1.3.1 Conventional theory of neurotransmitters and their receptors

Since neurotransmitters have been identified as mediators for neuronal communication in the last century, scientists devoted themselves to the research on the relations between the neuropsychological diseases and neurotransmitters. To date, it is widely believed that stress-related mood disorders are caused by a weakened function of serotonin, dopamine and noradrenalin (For a review, see Weiss and Coccaro, 1997; Curzon, 1988; Yehuda et al., 1988; van Heeringen, 2003). Therefore the classic antidepressants and antipsychotics target those monoaminergic systems. In addition, amino acid transmitters including glutamate and γ-aminobutyric acid (GABA) have attracted more and more interest in recent years as a target for a variety of diseases triggered by stress, including pain, sleep disorders, anxiety or mood disorders and substance abuse (For a review, see Lenoard, 2007; Skilbeck et al., 2010). In contrast to occupation of 5% of total synapses in the brain by monoamine transmitters, the excitatory glutamate and inhibitory GABA transmitters occupy at least 50% of synapses (For a review, see Lenoard, 2007). It also has been identified that these two regulatory transmitters are located in hypothalamic neurons (Boudaba et al., 1996, 1997). Evidence reveals that the interaction between the HPA axis and the amino acid transmitters system contribute to depressive diseases (For a review, see Gao and Bao, 2011).

1.3.2 Genetic studies

As in human studies mentioned above, genetic predisposition underlies the effects of early life stress. Because not only genetic heredity but also the variants induced by environment are associated with stress-related disorders, it is difficult to identify single genes responsible. Based on conventional molecular findings, it has been screened that glucocorticoid receptor (NR3C1), monoamine oxidise A (MAOA), glutamate receptor, CRF receptor-1 and CRF binding-protein encoding gene expression levels are associated with major depressive disorder and/or prediction of the response to antidepressant treatment (For a review, see Kupfer et al., 2011). Many studies identified some vulnerable genes, for example, serotonin transporter-linked polymorphic region (5HTTLPR), FKBP5, TREK1, MAOA, catchol-O-methytransferase (COMT) gene and dopamine receptor D4 (DRD4) gene, which are more susceptible to stressful environments (For a review, see Belsky et al., 2009).

1.3.3 Neuroimaging studies

Since the1990s, functional neuroimaging techniques have been widely used in emotional-related studies. A meta-analysis study of functional neuroimaging on depressive patients reported that although limited overlap is found by using different imaging methods, there still is consistently reduced activity in some regions including the dorsal/pregenual anterior cingulate, the middle frontal gyrus, the insula and the superior temporal gyrus at rest in depressed subjects and increased activity in certain subcortical and limbic regions (Fitzgerald et al., 2008). Some imaging studies also indicate that there is an abnormally increased activation in the amygdala, the ventral striatal and the mPFC of major depressive disorder subjects after negative emotional stimuli, and the depressive symptoms in older adults accompany reduced volume of the ACd (For a review, see Kupfer et al., 2011). In late life depression, the volumes of the ACd, inferior and superior medial frontal subregions are reduced (For a review, see Sexton et al., 2012). In contrast, in the measurement of gray matter, one study reported an increase in gray matter in the ACd. The hippocampus, as the most frequently studied region of interest, in 7 out of 15 comparison studies has been found to show a reduced volume of the hippocampus in depressed patients. Furthermore, depression-related contractions have been identified in bilateral anterior CA1-CA3 subfields, subiculum and dentate gyrus. Correlating the evidences from clinical and animal studies, it has been suggested that the reduction of hippocampal volume results from the increased glucocorticoid levels, which repress the dendritic development and inhibit neurogenesis (For a review, see Sapolsky, 2000).

In rodents, a previous study from our lab used using the 2-Fluoro-deoxyglucose method to investigate brain activity during parental separation stress, and results show that early life stress leads to a widespread reduction of brain activity compared to control animals (Bock et al., 2012).

1.3.4 Dendritic remodeling

Neuroimaging studies indicate that the reduced volume of the brain is a consequence of dendritic remodelling as the retraction of the neuronal structure is associated with chronic stress (Gorman and Docherty, 2012). Since mild stress during the early life induce an adaptation in certain brain regions, it is assumed that the adaptation may be also reflected by dendritic remodelling/synaptic structure alternation in these regions, such as the mPFC and in the hippocampus. In addition to MS-induced dendritic changes mentioned in chapter 1.2.1. Evidence from other stressed models also show that chronic stress induces retraction and lessen arborisation of apical dendrites in pyramidal neurons of the mPFC (Radley et al., 2004; Liston et al., 2006) and the hippocampus (Maria, 1996). In adulthood, a significant decline of dendrites that accompanies increased anxiety-like behaviour are induced following chronic restraint stress (McEwen et al., 2012). Furthermore, a number of evidences show that morphological alternations in spines correlated with maturation of synaptic physiology and synaptic plasticity (Harris et al., 1992; Yuste and Bonhoeffer 2001; Goldwater et al., 2009).

The original concept of synaptic plasticity can be expressed by Donald Hebb's famous postulate: *When an axon of cell A is near enough to excite a cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased—Donald Hebb 1949.* To date, a large number of molecules involved in synaptic plasticity have been identified, which underlie dendritic structural remodelling (Tsien et al., 1996; Bozon et al., 2002; Davis et al., 2003; Gareth et al., 2004; Tzingounis et al., 2006; Guan 2009; Huntley 2012; Lesch and Waider 2012). In our previous studies, two synaptic plasticity targets, activity-regulated cytoskeleton associated protein (Arc/Arg3.1) and early growth response protein 1 (Egr1/Zif268) had also been investigated, and it has been found that these two synaptic plasticity factors are regulated by early life experiences (Thode et al., 2005; Bock et al., 2005b). Our ongoing project still focuses on these two targets.

1.3.5 Synaptic plasticity genes Arc and Egr1

Arc protein is a protein highly related with neuronal structures, it is locally translated in spines and takes a crucial role in synaptic plasticity and memory (Tzingounis and Nicoll, 2006; Alme et al., 2007; Shepherd et al., 2011). Stress is reported to regulate Arc protein levels and the sustained translation of new Arc mRNA is required for long-term potentiation (LTP) consolidation and memory consolidation (Molteni et al., 2008; Elizalde et al., 2010), while the induction of LTP is accompanied with structural remodeling of dendritic spines and increased Arc protein expression in dendritic spines of dentate gyrus (For a review, see Yuste and Bonhoeffer, 2001; Rodríguez et al., 2005; Bramham et al., 2008). However, some studies reported that an activation of Arc induced by stress is restricted to telencephalic areas in the rat brain, while the most intense changes have been found in the PFC but no change was recorded in diencephalic and brainstem areas (Ons et al., 2004; Mikkelsen and Larsen, 2006; Schiltz et al., 2007). Our previous study is consistent with those findings and found that Arc mRNA was induced in subregions of the PFC equivalent in domestic chicks by early life emotional experiences, which are presumably directly related to learning-induced neuronal and synaptic alterations (Bock et al., 2005b). Moreover, some studies indicate that stress-induced Arc expression is controlled by glucocorticoids (Mikkelsen and Larsen, 2006; Schiltz et al., 2007).

Egr1, another target in the present study, is a marker of brain activity and neuroplasticity. More than 20 years ago. Egr1 has been reported to be associated to synaptic plasticity (Cole et al., 1989; Wisden et al., 1990). Our previous study also observed that Egr1 transcription was elevated in the forebrain following an acoustic imprinting stimulation in early life (Thode et al., 2005). Indeed, a number of studies have shown that environmental stimuli including learning and stress induce Egr1 expression (for a review, see Davis et al., 2003; Knapska and Kaczmarek, 2004). Moreover, Jones and colleagues (2001) found that Egr1 expression is required for consolidation of memory.

Notably, both Arc and Egr1 gene expressions are activated by mitogen-activated protein kinase (MAPK) pathway and occur rapidly and transiently after stimuli (For a review, see Guzowski 2002; Davis et al., 2003). Egr1 itself is a transcription factor, which remains in nucleus and directly regulates other gene transcriptions (James et al., 2005), including Arc gene transcription (Li et al., 2005), whereas Arc mRNA is transported to the dendrites and directly exerts on synaptic structure (Guzowski 2002). Although Arc and Egr1 are considered to be involved in the synapses' structural changes and processing of learning and memory, the

epigenetic mechanism that directly regulates gene expression of Arc and Egr1 remains unclear.

1.4 Epigenetics

1.4.1 Overview of epigenetics

Epigenetics is a research on mechanisms above the gene sequences. In the last decade, the concept of epigenetics has developed as "The sum of the alterations to the chromatin template that collectively establish and propagate different patterns of gene expression and silencing from the same genome" (Allis, et al., 2009, Chapter 3). The basic unit of the chromatin is the nucleosome, which is composed of ~147bp of DNA wrapped around an octamer of histones (H2A, H2B, H3 and H4); while Histone H1 links the nucleosome and functions in the compaction of chromatin into high order structures, which comprise chromosomes. The activity of gene transcription depends on the states of the chromatin, that is the euchromatin or heterochromatin. Activated chromatin is in an open state with relaxed nucleosome arrangement (ie. euchromatin) where DNA is accessible to the transcriptional factors (TFs) or chromatin associated proteins. Conversely, inactivated chromatin is in a condensed state where DNA is packaged and inaccessible (Allis, et al., 2009 Chapter 3). Thus, chromatin remodelling is considered a potential mechanism that underlies changes in gene transcriptions induced by environmental stimuli, which further result in physiological and/or pathological changes.

Most studies on chromatin remodelling investigate DNA methylation and histone modification. DNA methylation and histone modifications have been well-characterized and applied in oncology. DNA methylation is the first recognized epigenetic modification and is catalyzed by DNA methyltransferases (DNMTs). It occurs at the CpG sites (cytosine-phosphate-guanine dinucleotides), which are rich in promoter regions and non-coding genomic DNA. It has been shown that methylated CpGs are primarily in heterochromatin. In contrast, CpGs in euchromatin remain unmethylated (Allis, et al., 2009 book, Chapter 3). It has been indicated that DNA methylation is critical for gene silencing and chromatin structure.

Another factor that regulates chromatin remodelling is histone modification, which is mostly embodied by acetylation, methylation, phosphorylation and ubiquitylation. Those modifications regulate chromatin formations and activate or silence gene transcription. For an overview of different modifications indentified on histones see Table 1 below (Adapted from Kouzarides, 2007). Histone modification occurs on the amino acid tail, which extends from

the globular octamer region. The mechanisms underlying the function for each modification are not clear. It is widely believed that histone acetylation occurring at lysine residues of histone H3 and H4 activates gene transcription via neutralising the positive charged lysines binding to the negative charged DNA and opens the condensed chromatin that allows TFs bind to DNA promoter regions. In contrast, the functions of other histone modifications can be bi-directional. For example, research suggests that di- or tri-methylation of histone H3 at lysine 4, and phosphorlation of histone H3 at serine 10 also activate gene transcription, whereas di-or tri- methylation of lysine 9 or 27 on histone H3 silence the expression (Allis, et al., 2009, Chapter 3).

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Table 1: Overview of Histone Modifications

With the development of epigenetics research, further classes of modifications and their relevant enzymes are being identified (Kouzarides, 2007). As displayed in Table 1 above, acetylation exclusively occurs on lysine residues and the effect on the transcription is activation. So far, acetylation is one of the most well-studied histone modifications. Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are enzymes to acetylate and deacetylate lysine residue respectively.

In many cases, the epigenetic effects do not result from DNA methylation or histone modifications alone. For example, it has been suggested that there is a cross-talk between DNA methylation and histone modifications (For a review, see Vaissière et al., 2008). HDACs can interact with DNMT and integrate DNA methylation and histone modifications (Liu, et al., 2012). Conversely, methylated CpG islands can also recruit methylated DNA-binding proteins, which interact with HDACs and other proteins to mediate gene expression (For a review, see Klose and Bird, 2006).

1.4.2 Epigenetic studies in stress

There are five factors in the early environment during the development, which shape the epigenetic profiles and result in the alternations in later life. The five factors are nutrition, behavior, toxicants, stochasticity and stress (Faulk and Dolinoy, 2011). The present study focuses on early life stress effects on epigenetics and plasticity in later life.

1.4.2.1 DNA methylation and stress

There is increasing evidence suggesting that DNA methylation is regulated by stress. For example, early life stress increases the expression of arginine vasopressin (APV), and this increased expression is associated with DNA hypomethylation on the regulatory region of AVP, (Murgatroyd et al., 2009). Postmortem and animal studies indicate that the decreased levels of BDNF induced by stress may result from DNA hypermethylation on the promoter region of BDNF (Keller et al., 2010; Roth et al., 2011). On the other hand, administration of DNA methylation inhibitors shows an antidepressant-like effect that may be associated with increased BDNF levels (Sales et al., 2011). In contrast, most DNA methylation studies in stress-related models focus on factors of the HPA axis. A recent rodent study found that prenatal stress increases DNMTs mRNA levels in the frontal cortex and the hippocampus of offspring, and DNMTs binding to GAD67 promoter of GR indicate that decreased GR expression may be induced by DNA methylation (Matrisciano et al., 2012). The enhanced CRH transcription after maternal deprivation is associated with a decreased DNA methylation on the CRH promoter (Chen et al., 2012). Postmortem studies found a reduced hippocampal GR expression in suicide victims with a history of childhood abuse and further investigation found that the reduction of hippocampal GR expression is associated with DNA methylation in neuron specific exon 1_7 GR promoter (NR3C1) (McGowan et al., 2009a; Labonte et al., 2012). Interestingly, increased DNA methylation on NR3C1 is also observed in leukocytes of healthy subjects who reported on their childhood adversity and this change is correlated with a reduced response to CRH test (Tyrka et al., 2012). Also, prenatal stress increases DNA methylation on promoter of GR receptor NR3C1 in human umbilical cord blood samples (Mulligan et al., 2012).

However, those associations between DNA methylation and expression of NR3C1 are not always observed. One rodent study observed that MS during the early life induced alterations of neurochemicals and behaviours, as well as increased the plasma corticosterone level, whereas it fails to find a change in DNA methylation on Nr3c1 (Daniels et al., 2009). In addition, it's worthy noting that maternal care is a critical factor in regulation of DNA

methylation (Weaver et al., 2004, 2005; Meaney and Szyf 2005). Alison Rowan concludes that "the epigenetic mechanism that underpins these differences involves changes in the methylation state of the hippocampal GR promoter: in animals that have received high levels of maternal care, this promoter is hypomethylated, whereas lower levels of maternal care are associated with hypermethylation of this promoter" (Rowan, 2006).

1.4.2.2 Histone acetylations, stress and neuronal plasticity

There are two groups of enzymes, regulating the level of histone acetylation, HATs and HDACs. In the last decade, a numerous studies found that the alterations of histone acetylations are associated with aging and neurological diseases (Tsankova et al., 2004; Peleg et al., 2010; Castellano et al., 2012; Gräff et al., 2012; Soliman et al., 2012). The application of HDAC inhibitors has shown a promising effect on treatment of neurological diseases, for example Rubinstein-Taybi syndrome, Rett syndrome, Huntington's disease, and Friedreich's ataxia (For a review, see Sananbenesi and Fischer, 2009). HDAC inhibitors also exert antidepressant actions in mice (Covington et al., 2009). In a stress-related study, it has been observed that the typical anti-depressant imipramine shows the ability of inhibiting HDAC (Tsankova et al., 2006). However, hyperacetylation does not always play a positive role in the development. It is reported that the neurotoxic-induced neurodegeneration is relevant to hyperacetylations (Song et al., 2010). It is assumed that in histone acetylation as well as in any other drug, dose plays a crucial role. Thus, the extent of histone acetylation may be relevant for a positive or negative effect.

A very limited number of clinical studies clarify the role of HDACs in stress-related depressive disorder patients. One study investigated 11 subtypes of HDAC (HDAC1-11) in the blood samples of depressive disorder patients, the results showed that HDAC2, 4, and 5 mRNA were increased during the depressive episodes, whereas HDAC6 and 8 mRNA were found decreased in comparison to control (Hobara et al., 2010). Although there is no evidence indicating the function of each subtype of HDACs or which gene transcription is possibly associated with which HDAC, different subtypes of HDACs may regulate histone acetylations in different residues and further mediate the different gene transcriptions. For example, a study in rats have found that histone H3 acetylation increases transiently in the hippocampus after exposure to fear conditioning paradigm, but has failed to observe the similar change of H4 acetylation (Levenson et al., 2004). Similar findings were observed in social defeated rats where histone H3 but not H4 was increased by repeated social defeat (Hollis et al., 2010). Recently, Hollis and colleagues (2012) grouped the animals as high responders (HR) and low

responders (LR) based on their activity after exposure to mild stress, and interestingly they found that HR rats had higher acetylation on H3K4 in the hippocampus than LR rat had. However, social defeat decreased acetylation of H3K4 in HR rats but increased it in LR rats, and both groups after social defeat showed decreased histone H4 acetylation accompanied by no significant changes of HDACs mRNA expression in the hippocampus. In the forebrain, however, another recent study using a stress-susceptible mouse strain found that chronic MS decreased HDAC1, 3, 7, 8 and 10 mRNA expression in the forebrain, which is accompanied by an increase of histone H4 acetylation (Levine et al., 2012).

In the introduction (1.2 and 1.3), it was already mentioned that MS as an early life stressor results in behavioural manifestation, morphological changes, as well as the associated molecule alterations in the brain. The stress-induced long-term neuronal changes are possibly regulated by epigenetic mechanisms, which interfere with synaptic development and maturation. There is increasing evidence suggesting that histone acetylation plays an important role in synaptic plasticity, cognition and many psychological diseases (Levenson and Sweatt 2005; Gräff and Mansuy 2008; Hsieh and Eisch 2010; Day and Sweatt 2011; Mcclelland et al., 2011; Gräff et al., 2012). Studies in stressed models demonstrated that repeated MS in early life decreased HDACs mRNA expression and increased histone H4 acetylation in the forebrain neocortex in adulthood (Levine et al., 2012), and repeated social defeat resulted in a quick elevation of histone H3 acetylation in the hippocampus and rats after exposure to stress showed a long-term depressive-like behaviour (Hollis et al., 2010). Moreover, it was found that over-expression of HDAC2 in hippocampal pyramidal neurons decreased acetylation of H4K12 and H4K5 accompanied by impaired memory formation, and those results were confirmed by using HDAC2 knockout mice (Guan et al., 2009). Further investigation in dendritic structure found that the spine density was decreased in CA1 and DG of the hippocampus with over-expression of HDAC2, whereas increased in CA1 of HDAC2 KO mice (Guan et al., 2009).

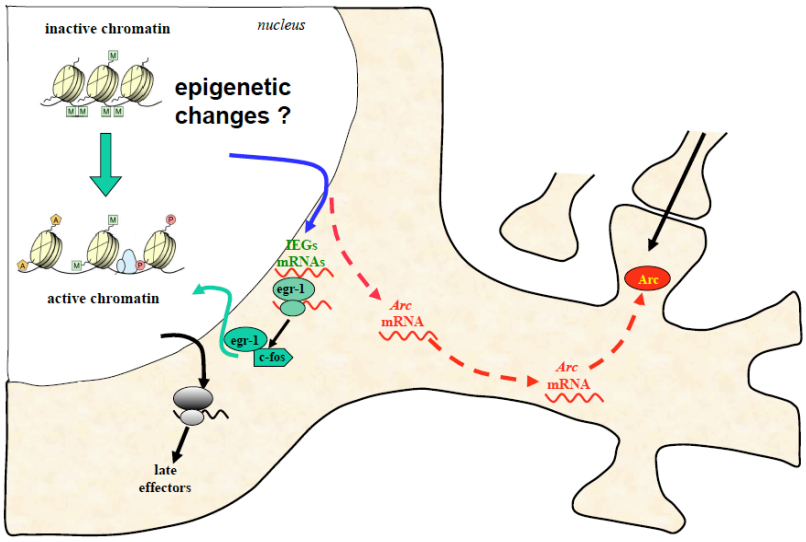
All in all, numerous studies in both, humans and animals, have shown that early life stress influences the neural circuit and behavioural function through life via epigenetic machinery. However, few studies of histone acetylation identified gene targets, such as BDNF, GR, CRF and RAS-MAPK-CREB signalling, which attracted the attention in stressed models (For a review, see Levenson and Sweatt 2005; McClelland et al., 2011; McEwen et al., 2012; Sun et al., 2012). Also the evidence to reveal whether epigenetic changes are associated with the neuronal plasticity is very limited. The present study investigated in the associations between

histone acetylations and neuronal plasticity-associated genes, as well as the long-term behavioural and neuron structure alternations.

1.5 Aim of the Study

According to Meaney and Ferguson-Smith (2010) "Epigenetic states lie at the interface between environmental signals and genome, serving to govern dynamic changes in transcriptional activity through extra- and intracellular mediators, in a multistep process, the epigenetic templates attracts specific effectors that determine the responsiveness of specific genomic regions to environmentally induced intracellular signalling pathways, thus leading to more stable effects on the potential for transcriptional activation and variation in neuronal function". It is important to note that the establishment of a stable inherited epigenetic state cannot occur without the first dynamic step (Dudley et al., 2011). So far, most studies focus on long-term epigenetic changes after stress and their association with synaptic structure and behaviours, whereas much less is known about early life stress induced early changes in gene expression and their regulation by epigenetic mechanisms.

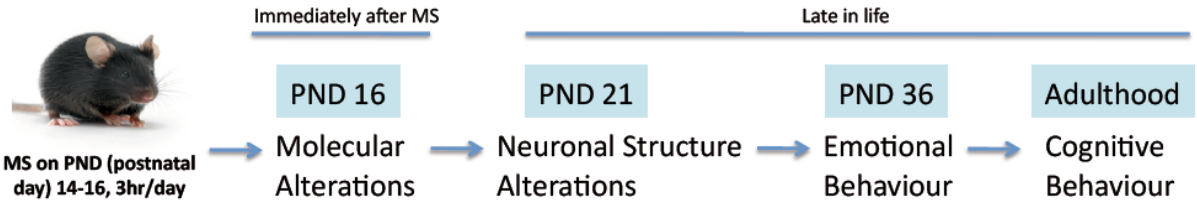
In previous studies, we were able to show that early-life experiences, such as the establishment of an emotional bond between mother and offspring induces significant increases in the gene expression of Arc and Egr1, two key molecules underlying learning and experience-induced synaptic plasticity in learning relevant brain structures, that were presumably directly related to learning-induced neuronal and synaptic alterations (Bock et al. 2005b; Thode et al. 2005). The present study applied repeated MS from PND 14-16 as an early life stressor and investigate histone acetylations, which may regulate the expression of Arc and Egr1 and are related to structural changes observed after early stress exposure (see Fig 1.2: Copyright, Jörg Bock).



The aim of the present study was to investigate the following questions:

1. Does repeated MS alter gene expressions of Arc and Egr1 in the hippocampus and the PFC immediately after stress?
2. Does repeated MS alter histone acetylations in the hippocampus and the PFC immediately after stress?
3. Do stress-induced gene expressions of Arc and Egr1 correlate with histone acetylations?
4. Are presumed molecular changes immediately after MS accompanied with neuromorphological alterations late in life?
5. Does MS induce emotional and cognitive behavioural changes late in life?

The time windows of investigations are summarized in the following scheme:



2. Methods

2.1 Animals and Maternal Separation Paradigm

C57BL/6 mice were used in all experiments and housed on a 12-hour light-dark cycle with access to food and water *ad libitum*. Following delivery of the pups the home cages were not cleaned during the first 16 postnatal days in order to avoid stress for the dams and pups due to handling and exposure to clean cage bedding. The newborn mice were culled to 6 or 8 per litter (male/female ratio was 3/3 or 4/4) and housed together with their mother. The day of birth was defined as PND 0 and entire litters were randomly assigned to either MS or control group. From PND 14 to 16, pups were separated from their mother and isolated individually in an 8-cube holding box (13cm x 13cm for each cube, covered with soft paper bedding) for 3 hours (10:00 AM-13:00 PM), where they could hear and smell each other, but had no visual or direct physical contact to their siblings. The pups were returned to their mother until to be sacrificed at different time points depending on experiments. All animals were handled in accordance with the European Communities Council Directive of November 1986 (86/609/EEC). In the present study, only male mice were investigated.

2.2 mRNA Expression

Male mice were sacrificed at 3 different time points: (i) 0 min after the end of the last separation period. (ii) 30 min after the end of the last separation period, or (iii) 120 min after the end of the last separation period. Control mice were sacrificed at same age and similar time. The prefrontal cortex and hippocampus were dissected and immediately frozen in liquid nitrogen, stored at -80˚C. RNA extraction was performed using RNeasy plus mini kit (QIAGEN GmbH) as follows:

(i) Frozen tissue was homogenized in 600µl of buffer RLT Plus using a mortar and pestle, and centrifuged the lysate for 3 min at maximun speed.

(ii) The supernatant was removed carefully by pipetting, and transferred lysate into a genomic DNA Eliminator spin column. Flow-through was saved after centrifugation at maximal speed for 30 sec.

(iii) 70% ethanol was added to flow-through and mixed well by pipetting. Then 2 x 600µl of each sample was transferred to an RNeasy spin column and centrifuged at maximal speed for 15 min.

(iv) RNeasy spin column membrane was washed by adding 700µl of buffer RW1 and 500µl of buffer RPE respectively after centrifugation at speed ≥ 10000 rpm for 15 sec. Then, additional 500µl of buffer RPE was added to each column and centrifuged at maximal speed for 2 min followed by placing RNeasy spin column in a new 2ml collection tube and centrifuged at maximal speed for 1 min.

(v) RNeasy spin column was placed in a new 1.5ml RNase free collection tube and added 40µl of RNase free water directly to the spin column membrane, RNA was eluted after centrifugation at maximal speed for 1 min. RNA isolation quality was examined by measuring A260/A280 and A260/A230 using NanoPhotometer (IMPLEN)

RNA samples were reverse transcribed into cDNA by using high capacity cDNA reverse transcription kit including Rnase inhibitor (Applied Biosystems). Quantitative polymerase chain reaction (qPCR) was performed on a StepOnePlus Real-time PCR System (Applied Biosystems) by using TaqMan gene expression assays, including Arc (Mm00479619_g1, Applied Biosystem), Egr1 (Mm00656724_m1, Applied Biosystems), hypoxanthine phosphoribosyltransferase 1 (Hprt1) (Mm01545399_m1, Applied Biosystems) Arc and Egr1 mRNA levels were caculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) and Hprt1 was selected as an internal control. The final value was expressed as relative value to control group. The reaction assay for each well was prepared as follows:

1. cDNA preparation:

10x RT buffer	6µl
25x dNTP mix (100mM)	2.4µl
10x RT random primers	6µl
MultiScribe reverse transcriptase	3µl
RNase inhibitor	3µl
Nuclease-free water	9.6µl
Total RNA sample	30µl

The thermal cycling conditions for reverse transcription:

25˚C 10 min

37˚C 120 min

85˚C 5 min

4˚C ∞

2. Gene expression assay reaction preparation:

20x TaqMan gene expression assay (Arc/Egr1/Hprt1)	1µl
2x TaqMan universal master mix	10µl
RNase free water	5µl
cDNA template (1-100ng)	4µl
Total volume	20µl

The thermal cycling conditions for gene expression reaction:

50˚C 2 min

95˚C 10 min

95˚C for 15 sec, 60˚C for 60 sec, gave 40 cycles

Held in 60˚C

2.3 Western Blot

2.3.1 Nuclei protein extraction

Male mice were sacrificed at 30 min after the end of the last separation period. Control mice were sacrificed at same age and similar time. The prefrontal cortex and hippocampus were dissected and immediately frozen in liquid nitrogen, stored at -80˚C. A protocol for nuclear extracts (Abcam protocol with small modifications) was applied. The tissue (~15mg) was homogenized in 1ml of ice-cold PBS containing 5mM sodium butyrate followed by centrifugation at maximal speed for 5 min, and then the sediment was re-suspended in 1ml of Triton extraction buffer (TEB, 1x PBS containing 0.5% triton x 100, 2mM phenylmethysulfonyl fluoride, 0.02% NaN_3). The lysate was placed on ice for 10 min with gentle stirring followed by centrifugation at 6500g for 10min at 4˚C to spin down the nuclei. After that, the supernatant was discarded and the nuclei washed in TEB. To extract nuclear proteins the pellet was re-suspended in 0.2N HCl and incubated overnight at 4˚C. The supernatant was collected and aliquoted after 10min of centrifugation at 6500g at 4˚C on the next day, stored at -20˚C for further use.

Protein concentration was measured by using BCA protein assay kit (Novagen®, Merck) as follows: (i) Dilutions of BSA standards were prepared as required and BCA working reagent containing 200µl of BCA Solution and 4µl of Cupric Sulphate for each sample. (ii) 25µl of each standard or protein sample replicate were pipetted into individual wells of a 96-well plate and mixed well with 200µl of BCA working reagent on plate shaker for 30 sec. (iii)

Plate was covered and incubated reactions at 37˚C for 30 min and measure the absorbance at 562nm on a plate reader when plate was cool down to room temperature. (iv) Absorbance of the blank standard was subtracted from all other standard and protein samples. (v) Standard curve and the equation were analyzed by using Excel, and then protein concentrations were calculated.

2.3.2 Immunoblotting

10µg of the lysates of nuclei protein extraction were mixed with 4x dye and 1M DTT (100mM of final concentration), and compensated the running buffer to 30µl of final volume. Samples were heated at 95˚C for 5 min before subjecting to a 4-15% gradient SDS-PAGE gel (Bio-Rad) and then blotted to a PVDM membrane (0.2micron, Millipore, Germany) at 30V for 2 hours. The blots were blocked in Roti block (Roth, Germany) working solution for 1.5 hours followed by incubation with primary antibodies (anti-acetyl-histone H3, 1:10,000, Millipore; anti-acetyl-histone H4, 1:4000, Millipore; anti-nucleolin, 1:1000, Abcam, Germany.) for overnight at 4˚C. The blots were incubated in horseradish peroxidase conjugated anti-rabbit antibody (1:4000, 12-348, Millipore) for 1 hour after washing 4x 10 min with TBST. Membrane was washed 3x 10 min with TBST and 1x 5 min with TBS before covering with Luminata crescendo western HRP substrate (Millipore) for 2-5 min. The blot images were visualized using a Fuji dark room with CCD camera system (FUJIFILM, LAS-1000). Optical density was measured using ImageJ program version 1.41o. To control the sample loading, optical density was normalized to the nuclear control protein nucleolin.

2.4 Native Chromatin Immunoprecipitation (N-ChIP)-Quantitative Real-time Polymerase Chain Reaction (qPCR)

2.4.1 N-ChIP

Male mice were sacrificed at 2 different time points: (i) 0 min after the end of the last separation period, or (ii) 30 min after the end of the last separation period. Control mice were sacrificed at same age and similar time. The prefrontal cortex and hippocampus were dissected and immediately frozen in liquid nitrogen and stored at -80˚C. We applied a ChIP protocol as described before with some modifications (Matevossian and Akbarian, 2008). The frozen tissue was ground in liquid nitrogen, and the homogenized tissue was transferred into Eppendorf tubes containing micrococcal nuclease (MNase) (USB) reaction buffer. Incubation was performed at 37˚C for 7 min after adding 10U Mnase and the reaction was terminated by adding 0.5M EDTA. Then hypotonisation was performed as follows:

(i) Placed sample into a 15ml falcom tube containing 5ml of 0.2mM EDTA and 5µl of 0.1M PMSF followed by incubation on ice for 1 hour. Sample was vortexed every 10 min and 1M DTT was added at the end of incubation.

(ii) Sample was centrifuged for 10 min at 4000g / 4˚C, the supernatant was removed to a clean 15ml falcom tube and 100µl of prewashed protein A sepharose beads was added.

(iii) This solution was rotated for 30 min at room temperature to preclean the lysate followed by centrifugation at 4000g / 4˚C.

(iv) After hypotonisation, lysate was split into fresh Eppendorf tubes as groups of input (500µl), no antibody control (1500µl) and the antibody (1500µl).

(v) The input was stored at -20˚C until the protein digestion step.

(vi) Samples with antibodies (anti-acetyl histone H3, 06-599; anti-acetyl histone H4, 06-866; Millipore) and no antibody group were incubated at 4˚C overnight after adding 165µl of 10x FSB.

(vii) Antibody-specific complex was precipitated by adding 80µl of prewashed protein A sepharose beads for 1 hour at 4˚C followed by centrifugation at 2000g for 5 min.

(viii) Then, the complexes were washed using washing buffers (high salt buffer, low salt buffer, LiCl buffer and TE buffer).

(ix) After centrifugation at 2000g /room temperature for 5 min, specific antibody bound chromatin fragments were eluted from beads using 2x 250µl of 0.1M NaHCO3 containing 1% SDS (rotated at room temperature for 15 min per time).

(x) In the last stage, the proteins were digested at 65˚C overnight and on the following day DNA was extracted with phenol-chloroform and purified with MinElute reaction cleanup kit (Qiagen, Germany) as follows: (a) 0.5ml phenol-chloroform was added into the sample and vortex, the mixture was pipetted to a 15ml phase-lock gel falcom tube and centrifuged at 1500g / room temperature for 5 min. (b) The upper phase (~500µl) was added into a clean 2ml Eppendorf tube and vortex after adding 300µl of buffer ERC. (c) Added 50µl of 3M NaAc and vortex (the colour became yellow indicated PH was 5). (d) Placed a MinElute column in a 2ml collection tube and allowed sample flow through the column by centrifugation at 13000 rpm for 1 min. (e) Flow-through was discarded and the membrane was washed with 700µl of buffer PE, followed by centrifugation at 13000 rpm for 1 min. (f) Centrifuged for an additional 1 min at maximal speed followed by placing the column in a clean 1.5ml Eppendorf tube, added

21µl of buffer EB directly on the membrane of column, allowed it stand for 1 min, then DNA was eluted after centrifugation at 13000rpm for 1 min.

Before the method was established, in order to optimise PCR efficiency, size of DNA fragment was estimated in this step. DNA was extracted as described above, and then amplified with the random primers (Primer A: 5'-GTAACGGAGTCAGAATTC NNNNNNNNN-3'; Primer B: 5'-GTAACGGAGTCAGAATTC-3') by using conventional PCR (conditions followed below).

1. Sequenase reaction preparation:

Mix A

Sequenase buffer	2µl
Primer A (40pmol/µl)	1µl

Mix B

Sequenase buffer	1µl
10mM dNTPs	0.5µl
0.1 M DTT	0.75µl
0.5mg/ml BSA	1.5µl
T7	0.3µl
Distilled water	1µl

Mix C

T7	0.3µl
Distilled water	0.7µl

3µl of mix A and 7µl of DNA were added for each reaction and placed in thermal cycler and run the program as described below:

94°C 2 min

8°C 2 min

8°C 2 min (add 5.1ul of Mix B in the first cycle, and then add Mix C 1ul in the second cycle)

Ramp at 30% to 37°C 2 min

37°C 10 min

Giving two cycles, and then hold samples at 4°C.

2. Amplification buffer preparation:

10 x PCR buffer	2µl
Distilled water	14.4µl
dNTPs	0.4µl
Primer B (100pmol/µl)	1µl
Tag	0.2µl
Sequenase reaction sample	2µl
Total Volume	20µl

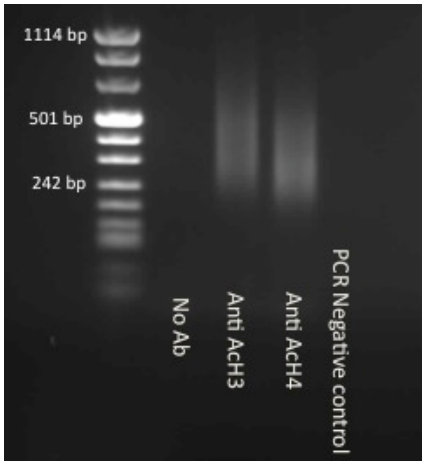
The thermal cycling conditions:

94˚C 2 min

94˚C for 30 sec, 40˚C for 30 sec, 50˚C for 30 sec, 72˚C for 60 sec, gave 30 cycles

72˚C 3 min

3. 5µl of PCR products was ran in 1.2% agarose gel. The ideal size of DNA fragment for ChIP-qPCR is between 200- 500 bp. (see the following image).



2.4.2 qPCR

Q-PCR was performed using TaqMan assays (Applied Biosystems, Germany), using primers specific for the promoter regions of Arc or Egr1 (TIB, for primer sequences, see Guan et al. 2009). Beta-globin (5'-TGACCAATAGCCTCAGAGTCCTG-3' and 5'-GAAGACCTGTCC TTTTATTCTTCACC-3', 6FAM-ACCTTGCTCCCCTTACC CCTCC-BBQ) was used as locus control region with low levels of histone acetylation (inactivated control gene). Each PCR reaction was run in triplicates for each sample. The immunoprecipitation data were normalized to input (ChIP-Input ratio) as an index of transcription.

qPCR reaction assay preparation:

DNase free water	6.5µl
2x TaqMan universal master mix	10µl
Forward primer (10pmol/µl)	1µl
Reverse primer (10pmol/µl)	1µl
Probe (10pmol/µl)	0.5µl
DNA Template	1µl

The thermal cycling conditions:

94˚C 10 min

94˚C for 15 sec, 58˚C for 15 sec, 72˚C for 15 sec, gave 50 cycles

Held in 4˚C

2.5 Corticosterone Measurement

Blood was collected in Serum-Gel Z tube (Sarstedt) after decapitation, immediately (0 min) after the last separation period. Blood from control mice were collected at same age and similar time. Samples were allowed to clot for 30 min before centrifugation for 5 min at 10000 x g at 20˚C. Serum was removed to a clean Eppendorf tube and stored samples at -80˚C. Corticosterone levels were measured using corticosterone rat/mouse ELISA kit (DEV9922, demeditec) and the procedures as follows:

(i) 10µl of each calibrator and sample were dispensed into appropriate wells (provided by demeditec) followed by the addition of 100µl of incubation buffer into each well.

(ii) 50µl Enzyme Conjugate was added into each well and incubated for 2 hours at room temperature on a mixer (>600 rpm).

(iii) The content of the wells was discared and the wells were washed 4 times with 300µl of Washing Solution. As much Washing Solution as possible was removed by blotting the microplate on towel paper.

(iv) 200µl of Substrate Solution was added to each well and incubated without shaking for 30 min in the dark followed by terminating the reaction using 50µl of Stop Solution to each well.

(v) The absorbance of each well was determined at 450nm in 5 mins.

(vi) Standard curve and the corticosterone concertrations of samples were read automatically by software (ScanIt Sofware V2.5).

2.6 Quantitative Analysis of hippocampal neuronal morphology

Male mice were decapitated on PND 21, the brains were rapidly removed and the unfixed brains were immersed in Golgi-Cox solution for 14 days. After the brains were sectioned at 150µm and processed and developed as described in previous studies (Bock et al. 2005a). For each animal (8 animals per experimental group) 3 neurons of the following neuron types were analyzed: (i) Pyramidal neurons located in the dorsal hippocampal CA3 subregion (ii) Granular neurons in the dentate gyrus. All neurons were using a computer-based neuron tracing system (NEUROLUCIDA®, MicroBright-Field, Williston, VT, USA), which allows the quantitative 3D analysis of complete dendritic trees. The following parameters for each reconstructed neurons were quantified: (i) proximal, medial and terminal apical dendritic length, and the number of spines on them respectively. (ii) apical dendritic complexity. (iii) Spine density in apical dendrites. The length of the dendritic trees was measured by tracing the entire dendrite. In addition to the calculation of total dendritic length a Sholl analysis (Sholl 1953) was performed to measure dendritic complexity by applying concentric rings, 50 µm distance apart from each other (Bock et al. 2011). Spine density was mesured in 5 fragments of each dendrite type (proximal dendrite, medial dendrite and terminal dendrite respectively). All measurements were performed by an experimenter who was unaware of the experimental condition of the animals.

2.7 Behavioural Tests

2.7.1 Forced swimming test (FST)

Control offspring (n = 16) from 4 litters and MS offspring (n = 19) from 5 litters were tested. Forced swimming was conducted on 2 consecutive days (pre-test on PND35, test on PND36). The mice were placed individually in a glass cylinder (28cm in height and 15cm in diameter) filled with water (22˚C, 19cm in depth) for 15 min on each day. Behaviours were recorded by using a video camera. Immobility was defined as floating without struggling or swimming, and only a minimal movement to maintain the head above water. The experimenter analyzing the test was blind to experimental condition of the animal.

2.7.2 Spatial learning

Prior to the spatial learning procedure, an radio frequency identification (RFID) transponder was subcutaneously injected in the interscapular area of each animal for automatic individual

recognition in the IntelliCage (NewBehavior AG, Switzerland), which allows the automatic assessment of spatial learning without stressful handing that may interfere with learning and memory functions. For details of the system see (Codita et al. 2010, 2012). The injection of RFID was performed under Isoflurane. 14 control and 15 MS adult subjects for each group (4 litters per group) were tested. The cage was equipped with four automatically operated corners, which contained water bottles. Siblings were placed in one cage. Spatial learning was assessed as follows:

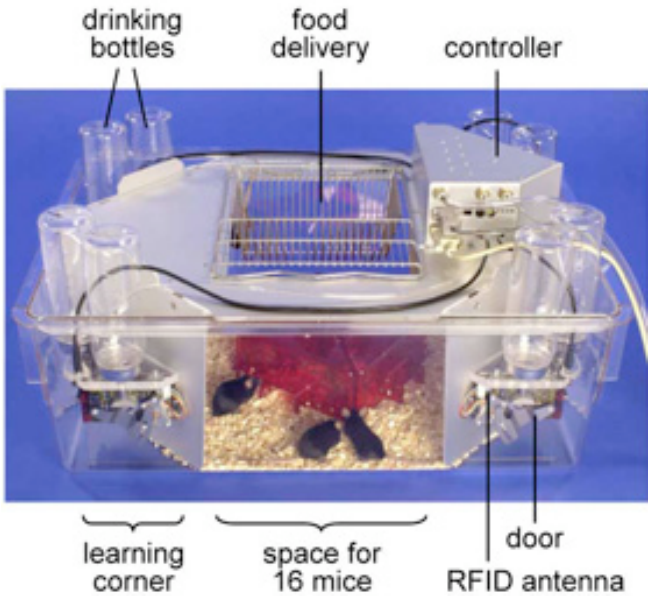
(i) 1 day free adaptation: all doors in each corner were open, and the mice had free access to water.

(ii) 3 days nose poke adaptation: doors were closed, and the mice had to learn to poke at the close door and wait for the door to open for water access.

(iii) 2 days place learning: same as described in (ii), but each mouse was assigned to one corner (correct), i.e. it gained access to water only when it poked at this door.

(iv) 4 days place learning reversal: same as described in (iii), but the rewarded corner was diagonally opposite to the one rewarded during place learning. The percentage of visiting the correct corners was analyzed.

The equipments for behavioural tests are shown in following pictures. (Left: FST, adapted from http://neurobehaviour.lunenfeld.ca/DEFAULT.ASP?page=Depression; Right: Spatial learning test in IntelliCage, adapted from http://www.neuroscience.ethz.ch/research/neural basis/wolfer/index)



2.8 Statistics

Two-tailed t test was applied in analysis of gene expression, western blot, ChIP qPCR data, histological data, forced swim test, serum corticosterone level and body weight. For spatial learning test, general linear model for repeated measures was applied, followed by two-tailed t test for further analysis of each day. All data were presented as means ± standard error of the mean (S.E.M). Significance was set at $p \leq 0.05$ for all data sets.

3. Results

3.1 Region-specific Enhancement of Gene Expression in Stressed Animals

In order to test Arc activity after MS, we examined Arc mRNA levels in PFC and hippocampus at 3 time points: 0 min, 30 min and 120 min after the last session of MS. To reduce the environmental effects, gene exression levels of MS mice were to the comparable gene expression levels of controls (ie. MS and control litters were born and raised during the same week).

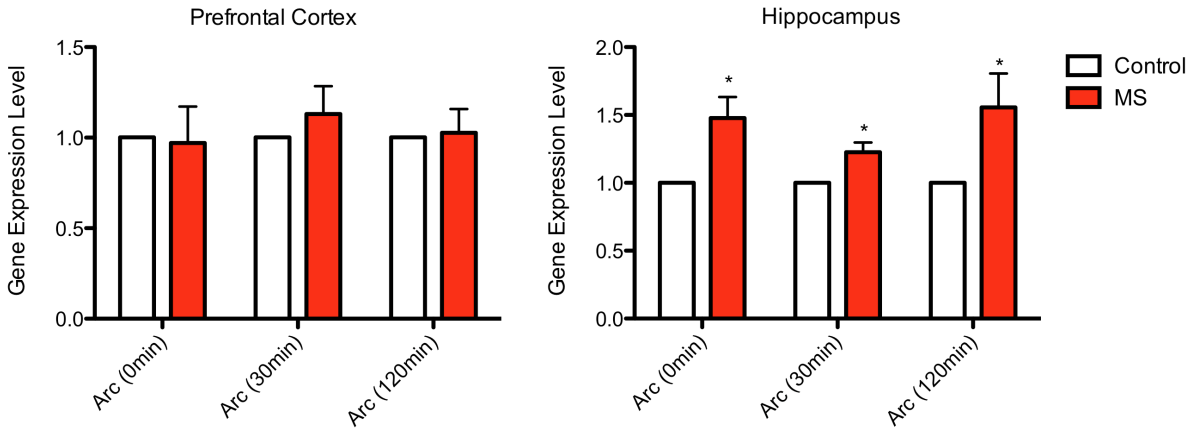


Figure 3.1: Arc Gene Expressions. (A) Arc mRNA in PFC: There is no significant difference in Arc mRNA expression in the PFC between control and MS groups at any time point over 2 hours after the last MS session: Arc (0min): p=0.884; Arc (30min): p=0.403; Arc (120min): p=0.839. **(B)** Arc mRNA in hippocampus: Arc mRNA is upregulated in the MS group at all experimental time points: Arc (0min): p=0.008; Arc (30min): p=0.005; Arc (120min): p=0.038. The sample size for Arc mRNA measurements is for 0min: n=9 per group at 0min; n=12 per group at 30min and 120min.

The results shown in Fig. 3.1 indicate that transcription of the Arc gene is activated in the hippocampus immediately after MS (p=0.008), and the activation of transcription remains significant at 30 min (p=0.005) and 120 min (p=0.038) after MS compared to controls. However, there was no activation of Arc gene transcription in the PFC at 0, 30 or 120 min (p=0.884, 0.403 and 0.839 respectively). From these results, it seems that MS has a greater impact in the hippocampus than in the PFC.

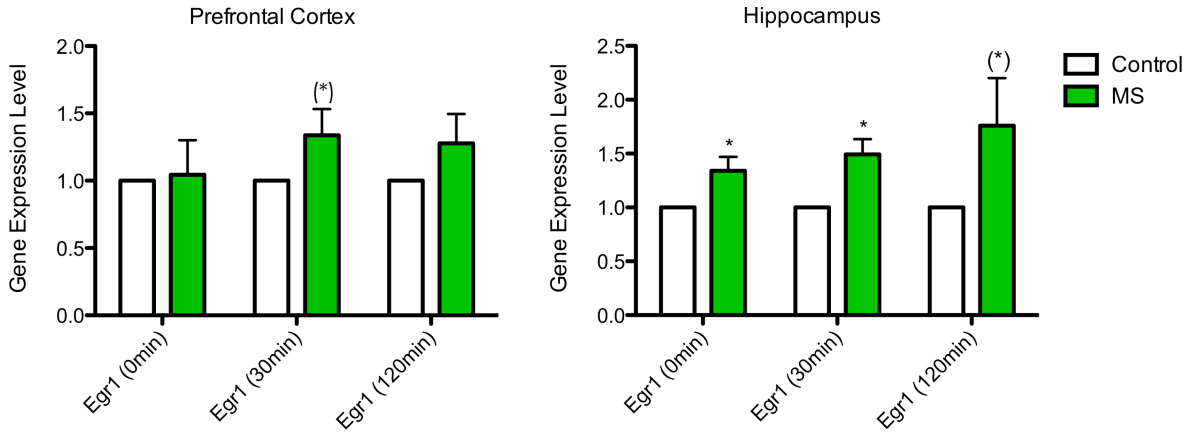


Figure 3.2: Egr1 Gene Expression. (A) Egr1 mRNA in PFC: There is no significant difference in Arc mRNA expression in the PFC between control and MS groups at any time point over 2 hours after the last MS session, tendency toward increased Egr1 mRNA is seen at the 30min time point: Egr1 (0min): p=0.864; Egr1 (30min): p=0.098; Egr1 (120min): p=0.213. **(B)** Egr1 mRNA in hippocampus: The level of Egr1 mRNA is significantly elevated at the 0min and 30min time points: p=0.019 and p=0.002 respectively. A non-significant tendency towards an increase was observed at the 120min time point (p=0.100). The sample size for Arc mRNA and Egr1 mRNA measurements is n=9 per group at 0min; n=12 per group at 30min and 120min. (* p<0.05, (*) p<0.1)

Egr1 gene expression levels in the PFC and hippocampus were also tested after MS at 3 different time points. The results demonstrate enhanced gene transcription of Egr1 in the hippocampus, but not in the PFC (see Fig 3.2). It can be seen that Egr1 mRNA levels are increased in the hippocampus at 0 min and 30 min after MS (p=0.019 and 0.002 respectively), whereas only a tendency towards an increase is observed at 120 min in the hippocampus of MS mice (p=0.100) compared to controls. In the PFC, Egr1 gene expression is similar to what was found in Arc gene expression. There are no significant changes in the activity of Egr1 transcription at any time point (p=0.864, 0.098 and 0.213 stands for 0 min, 30 min and 120 min, respectively). However, the activation of Egr1 transcription at 30 min shows a tendency toward an increase compared to controls.

The results of Arc and Egr1 gene expression indicate that our paradigm of MS may exert more significant effects on gene expression in the hippocampus, but few in the PFC.

3.2 Elevated Expression of Acetylated Histone Proteins in Stressed Animals

It is well known that acetylation of histone facilitates gene transcription. In order to measure the level of acetylated histone and to investigate whether MS induces histone acetylations, western blotting was used to detected acetylated histone H3 and H4 levels in the PFC and hippocampus, respectively. The acetylated level of histone H3 and H4 was analyzed only at 30 min after the last session of MS. As protein level alternations are not evident as soon as gene expression and histone acetyations are expected to occur before activations of their associated genes transcription, the 30 min post-MS time point alone was employed in detection of acetylated histone proteins.

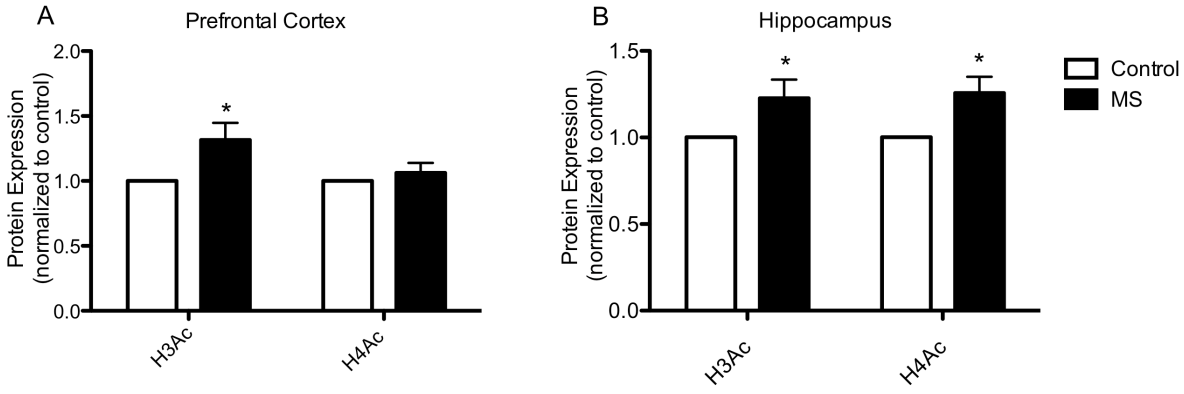


Figure 3.3: Protein Expression of Acetylated Histones. (A) Acetylation of histones in PFC: Acetylated histone H3 expression in the PFC is significantly increased in MS group: p=0.032 (n=8 per group), while there is no difference at acetylated histone H4 level: p=0.418 (n=8 per group). **(B)** Acetylation of histones in hippocampus: Both acetylated histone H3 and H4 are significantly elevated in MS group: H3Ac: p=0.050 (n=10 per group); H4Ac: p=0.013 (n=11 per group).

It can be seen that MS induces acetylations on histone H3 both in the PFC and hippocampus (see Fig 3.3, p=0.032 and 0.050, respectively). Elevation of pan-acetylations on histone H4 is observed only in the hippocampus after MS (p=0.013), and alternations of acetylated histone H4 in the PFC after MS were not detected (p=0.418). The pattern of protein alternations is similar to that of gene expression, that is, there is a more significant enhancement of acetylated histone levels in the hippocampus than that in the PFC following exposure to MS. However, as these factors were measured over a relatively short time scale, one cannot conclusively declare that MS does not induce significant alternations in the PFC or that any alternation in the PFC may be evident at a later stage than this in the hippocampus.

3.3 The Association between Histone Acetylation and Gene Expression

As described above, an activation of Arc and Egr1 gene transcription was observed in the hippocampus, and also increased acetylation of histone H3 and H4 was observed in the PFC and hippocampus after MS.. In order to investigate whether the activation of gene expression is correlated with the elevated acetylation of histone H3 and H4, ChIP-qPCR was used to detect whether the promoter region of Arc and Egr1 gene are contained in the DNA fragments, which were precipitated together with histone acetylations.

3.3.1 Histone H4 acetylation correlates activation of Arc and Egr1 genes, histone H3 acetylation correlates activation of Egr1 gene but not Arc gene in the PFC.

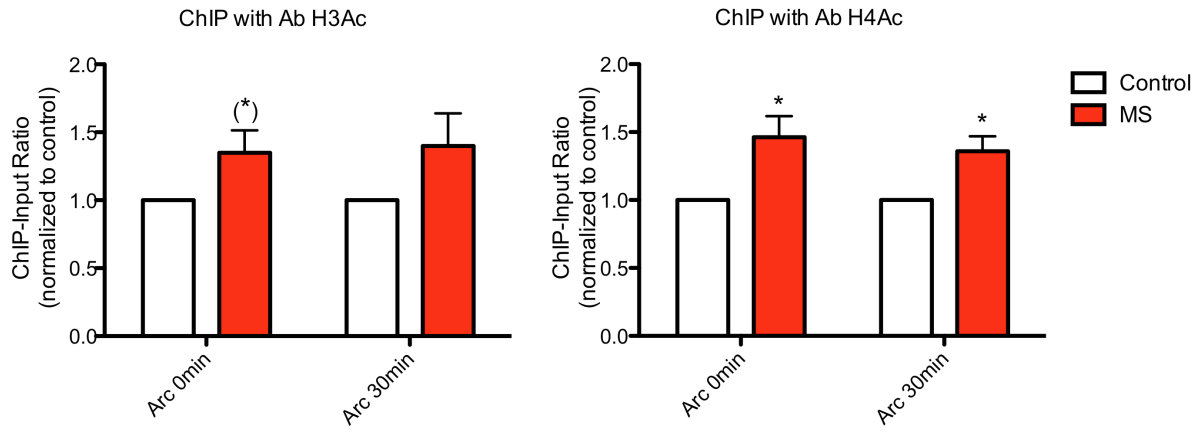


Figure 3.4: ChIP-Input Ratio at Arc Promoter Region in the PFC. (A) ChIP-Input ratio of histone H3 acetylation: There is no alternation in histone H3 acetylation correlating to the promoter regions of Arc gene 30 min after MS, whereas a tendency towards an increase is seen at 0min after MS: Arc (0min): p=0.079 (n=4 per group); Arc (30min): p=0.122 (n=7 per group). **(B)** ChIP-Input ratio of histone H4 acetylation: Increased histone H4 acetylation correlating with the promoter region of Arc is induced by MS: Arc (0min): p=0.007 (n=12 per group); Arc (30min): p=0.006 (n=8 per group). (*p<0.05, (*)p<0.1)

Although there was no alternation of Arc gene expression and acetylated histone H4 detected in the PFC, there is a significant elevation of acetylated histone H4 around Arc promoter region immediately after MS and this is still evident after 30 min (Fig 3.4, p=0.007 and 0.006 respectively). This suggests that acetylation of histone H4 may specifically occur around some genes, and the amount of modified histone is not detectable in western blot. In contrast, a significant elevation of acetylated histone H3 was observed in the PFC after MS, however, acetylation of histone H3 only shows a tendency towards increasing around the Arc promoter

region in MS animals at 0 min (p=0.079), and there is no significant difference at 30 min between MS pups and controls (p=0.122); which indicates acetylation of histone H3 is not correlated with Arc promoter region in the PFC.

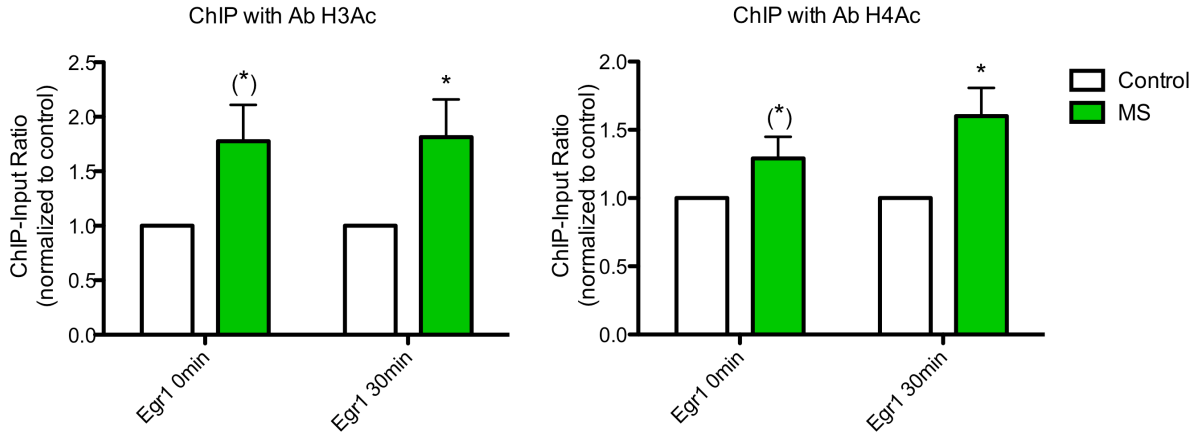


Figure 3.5: ChIP-Input Ratio at Egr1 Promoter Region in the PFC. (A) ChIP-Input ratio of histone H3 acetylation: The MS group shows a tendency towards an increased histone H3 acetylation correlating with the promoter regions of Egr1 gene immediately after MS, and the increase is significant at 30min: Egr1 (0min): p=0.059 (n=4 per group); Egr1 (30min): p=0.036 (n=7 per group). **(B)** ChIP-Input ratio of histone H4 acetylation. The alternations in histone H4 acetylation around Egr1 promoter region are as same as what we observed in histone H3 acetylation: Egr1 (0min): p=0.078 (n=12 for each group); Egr1 (30min): p=0.012 (n=8 for each group). (*p<0.05, (*)p<0.1)

The investigation of the association between activation of Egr1 gene transcription and acetylation of histones reveals that the acetylation of histone H3 and H4 around Egr1 promoter region is not significantly elevated in the PFC immediately after MS, however, a tendency towards increase can be seen (Fig 3.5, p=0.059 and 0.078, respectively). Thirty min later, the increase of histone H3 and H4 acetylations around Egr1 promoter region becomes significant (p=0.036 and 0.012, respectively), indicating that histone H3 acetylation correlates with activation of Egr1 gene transcription in the PFC, which may induce the mild elevation of Egr1 gene expression at 30 min.

3.3.2 Histone H4 acetylation correlates activation of Arc and Egr1 genes in the hippocampus

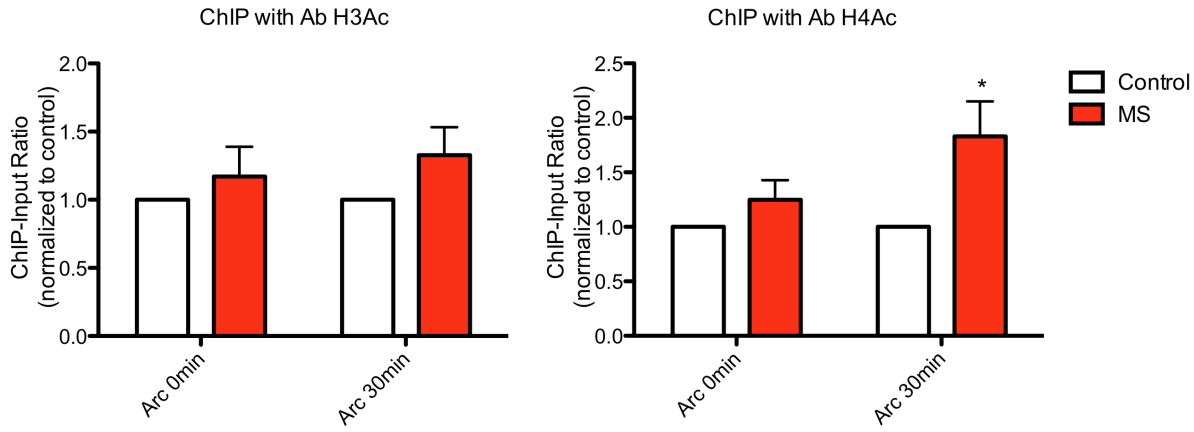


Figure 3.6: ChIP-Input Ratio around Arc Promoter Region in the Hippocampus. (A) ChIP-Input ratio of histone H3 acetylation: MS does not change histone H3 acetylation correlating to the promoter regions of Arc gene: Arc (0min): p=0.447; Arc (30min): p=0.126 (n=10 per group). **(B)** ChIP-Input ratio of histone H4 acetylation: Histone H4 acetylation correlating to the promoter region of Arc is increased only at the 30min time point: Arc (0min): p=0.184 (n=10 per group); Arc(30min): p=0.018 (n=11 per group).

In the hippocampus, there is a profound elevation in Arc gene expression and this lasts over 120 min after MS. In addition, both histone H3 and H4 show a significantly increased acetylation level. It was expected that significant increases in histone acetylations around the promoter region of Arc gene would be observed. However, the result only indicates that the activation of Arc gene expression is correlated with increased acetylation of histone H4 (Fig 3.6, p=0.018) but not histone H3 30 min after MS (p=0.126). Moreover, there is no alternation of histone acetylations around Arc promoter region immediately after stress (p=0.447 and 0.184 for histone H3 and H4, respectively).

Although acetylation of histone H3 is significantly increased in the hippocampus, it is not correlated with the activation of Egr1 gene transcription as it fails to induce the elevation of acetylated histone H3 around the promoter region of Egr1 (Fig 3.7, p=0.230 and 0.173 at 0 min and 30 min, respectively). In contrast, it can be seen that the increased acetylation of histone H4 occurs around the promoter region of Egr1 (p=0.046 and 0.015 at 0 min and 30 min, respectively), which implies a strong correlation between elevated acetylation of histone H4 and the activation of Egr1 gene transcription in the hippocampus.

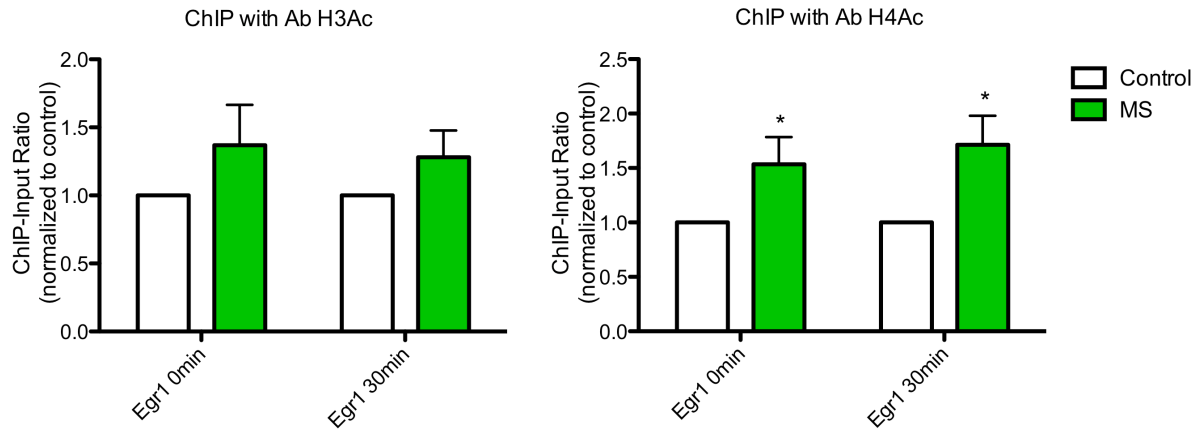


Figure 3.7: ChIP-Input Ratio around Egr1 Promoter Region in the Hippocampus. (A) ChIP-Input ratio of histone H3 acetylation: MS does not change histone H3 acetylation correlating to the promoter regions of Egr1 gene: Egr1 (0min): p=0.230. Egr1 (30min): p=0.173 (n=10 per group). **(B)** ChIP-Input ratio of histone H4 acetylation: MS significantly increases histone H4 acetylation correlating to the promoter region of Egr1 in the MS group: Egr1 (0min): p=0.046; Egr1 (30min): p=0.015 (n=10 per group).

3.4 Elevated Serum Corticosterone Levels in Stressed Animals

Corticosteroids are steroid hormones involved in stress response, and the normal release of corticosterone shows a circadian rhythm. In rodents maintained on a 12:12 hr light/dark cycle, concentration of serum corticosterone is at lowerest level at the onset of light phase (05:00), it increases during the light phase and reaches peak at the onset of dark phase (17:00) (D'Agostino et al., 1982). Our mice used in this experiment were sacrificed at 13:00-14:00, the normal range of serum corticosterone level between 11:00-14:00 in male mice is 47-159 ng/ml (provided by Demeditec Diagnostics GmbH).

In the present study, the blood samples were collected from decapitated mice during 13:00-14:00 (ie. immediately after MS for the MS animals). It is shown (Fig 3.8A) that the level of serum corticosterone is significantly elevated in MS group (p=0.004), and an approximately 2.5 fold increase was observed in MS compared with control mice. Fig 3.8B shows the individual data from each animal, it can be seen that all data from control animals falls within the normal range of serum corticosterone. Although the average serum corticosteronel level of MS mice is in the normal range, some MS animals show a higher level than the normal level after MS. The elevation of corticosterone level indicates MS is a stressful event for our experimental subjects.

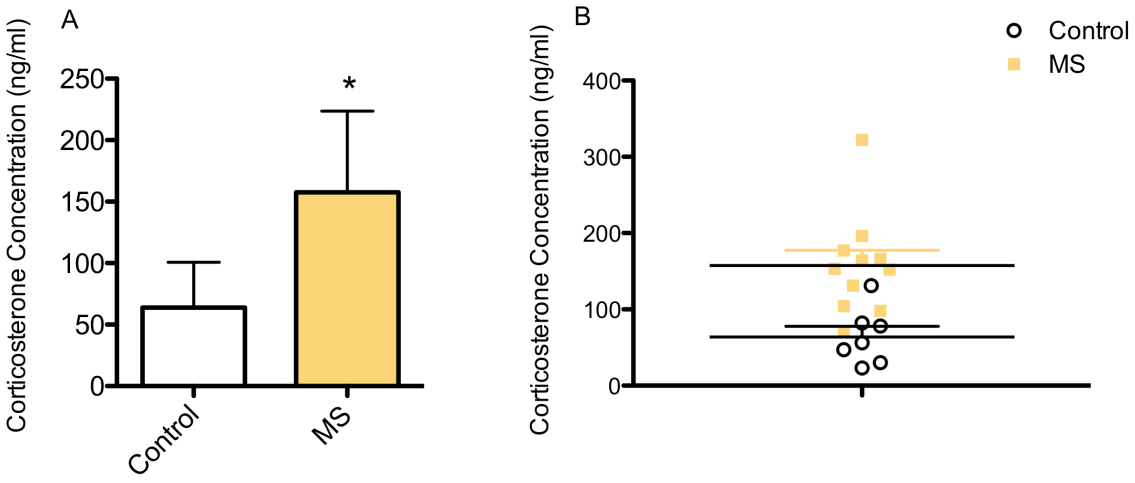


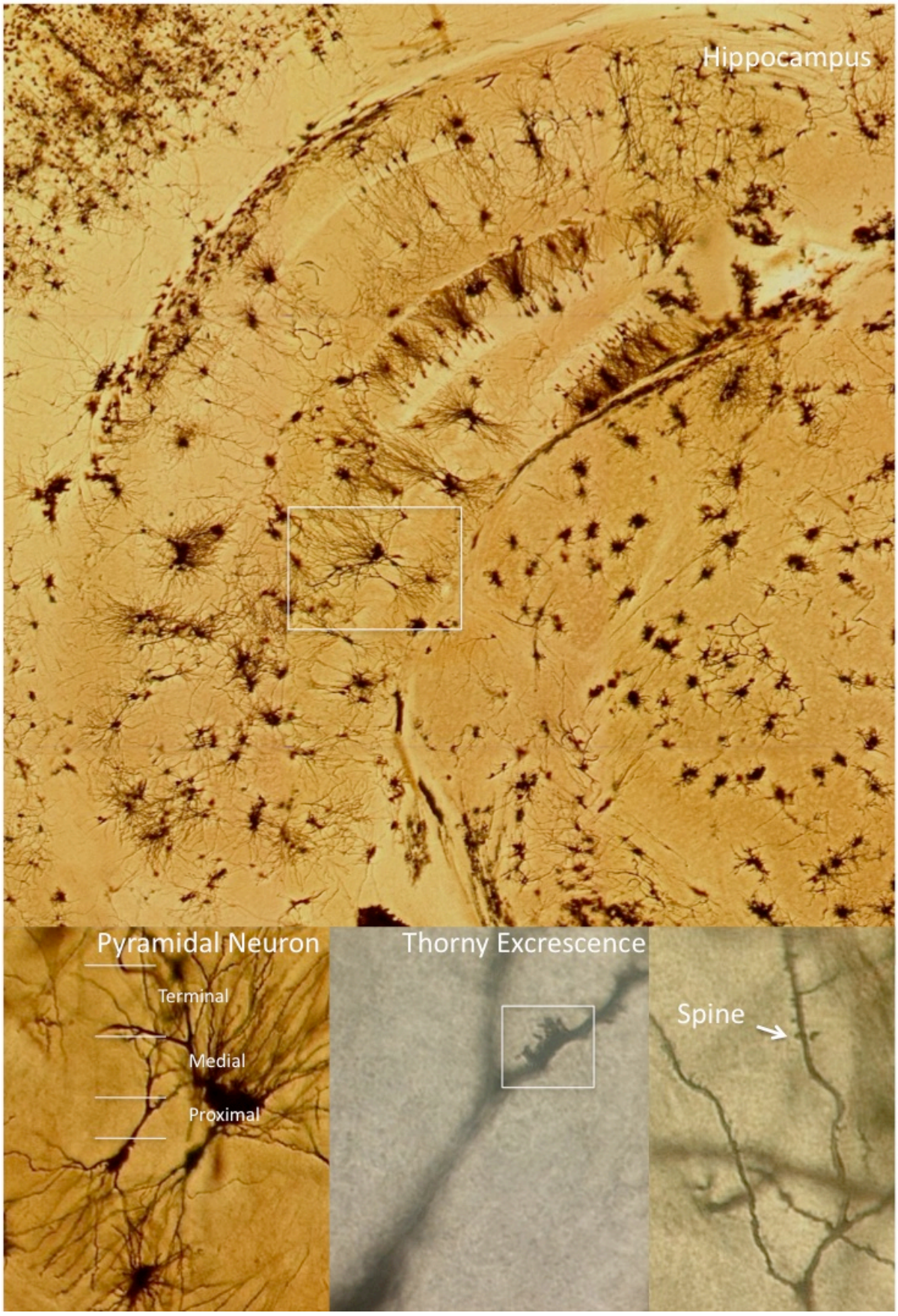
Figure 3.8: Serum Corticosterone Level. (A) Elevated serum corticosterone: Corticosterone levels significantly increase after MS (p=0.004, n=7 in control group, n=11 in MS group). **(B)** The pattern of serum corticosterone concentration for each animal.

3.5 Increased Apical Dendrite Complexity and Spine Number in Hippocampal CA3 Neurons of Stressed Animals

Since alterations in synaptic structure develop over several days after MS exposure, the subjects used in the present experiment were sacrificed 1 week after MS, on PND 21. In the analysis, 2-4 neurons for each animal were traced and an average value from each animal was analyzed using two-tailed t-test.

A massive cluster of spines is observed in proximal dendrites in the CA3, referred to as thorny excrescences. It has been reported that Thorny excrescences are regulated by stress and learning events (Stewart et al., 2005). Thus, we further analyzed spine density by distinguishing single spine and thorny excrescences separately (Picture 3.1). There was no significant difference in spine density observed between MS group and control groups (Fig 3.10B, p=0.172). Nor is there any difference in the density of thorny excrescences (Fig 3.10B,

p=0.779).



Picture 3.1: Representative Image of Golgi Staining. Top: Hippocampus; Bottom Left: Pyramidal neuron in CA3l; Bottom Medial: Thorny Excrescence; Bottom Right: Spine.

It is worth noting that not all proximal dendrites of pyramidal neurons in the CA3 region contain thorny excrescences. In the present analysis, thorny excrescences are observed in 4 out of 8 animals either in MS group or control group.

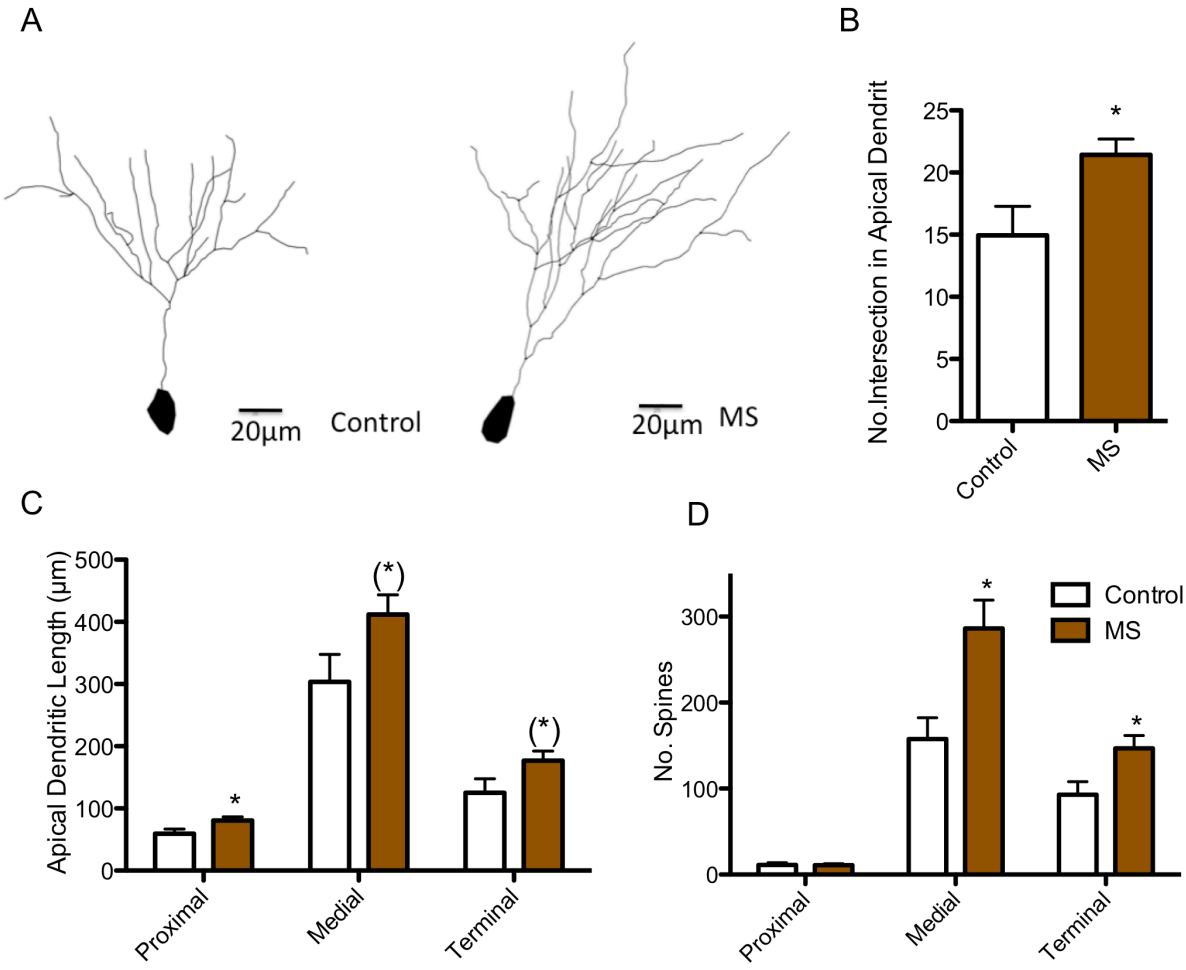


Figure 3.9: Increased Dendritic Length, Spines and CA3 Apical Dendrites Complexity. (A) Representative examples of three-dimensionally reconstructed apical dendrites of CA3 pyramidal neurons in the control and MS group. (B) MS significantly increases the complexity of CA3 apical dendrites (p=0.029, n=8 per group). (C) An increased CA3 proximal apical dendritic length (p=0.043) is observed in MS mice compared to controls, and a trend towards elongated medial and terminal apical dendrites (p=0.066 and p=0.077) in MS animals. (D) The number of spines in medial and terminal apical dendrites is increased (p=0.007 and p=0.024) after MS, whereas no change in proximal dendritic spines (p=0.96). (*$p\leq0.05$, (*)$p\leq0.1$).

Further, Sholl anaylsis reveals that MS induces a significant increase of dendritic intersection in apical dendrites of CA3 pyramidal neurons (Fig 3.9B, p=0.029), which indicates that a higher dendritic complexity and neuronal interaction network is formed after short period repeated MS (Fig 3.9B). In addition, a significant increase in CA3 proximal dendritic length is observed in MS mice (Fig.2C, p=0.04), while medial and terminal apical dendritic length show a trend towards increase (p=0.07 and p=0.08, respectively).

The spine is the region where the neuron interacts with other neurons. Thus, the spine genesis in dendrites is very important in neural plasticity. Our results show an increased number of spines in medial and terminal dendrites in MS mice (Fig.2E, p=0.02 and p=0.007), whereas there is no change in proximal dendrites (p=0.95).

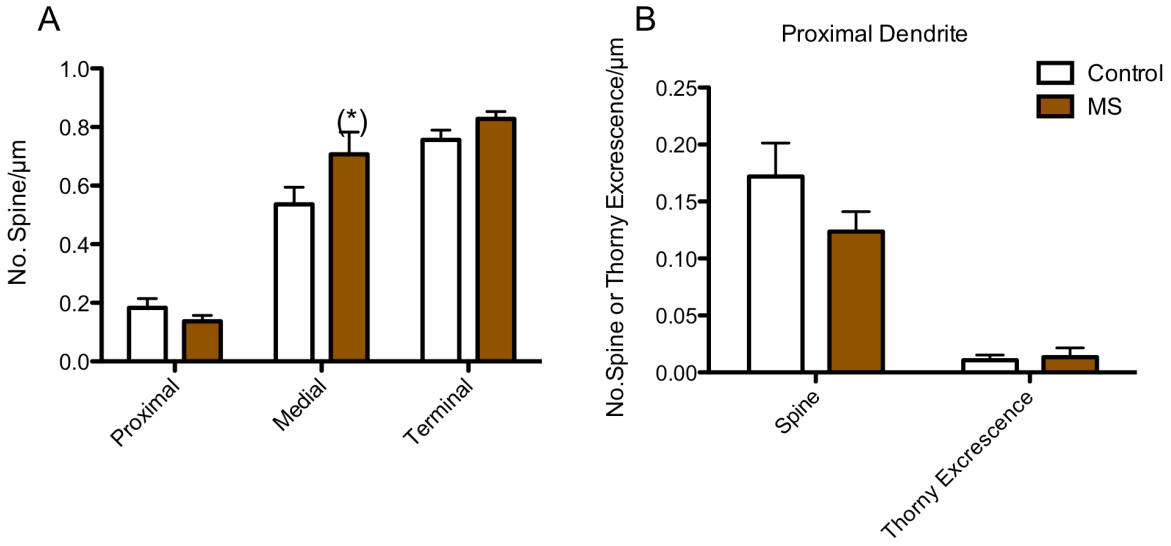


Figure 3.10: Spine Density in CA3. (A) Spine density in different distances to cell body: Spine densities remain unchanged after MS in proximal dendrites (p=0.245, n=8 per group) and terminal dendrites (p=0.105, n=8 per group), while there is a tendency towards increase in medial dendrites (p=0.097, n=8 per group). **(B)** Density of spine and thorny excrescence in proximal dendrites: There is no difference in either spine density or Thorny excrescence density in proximal dendrites (p=0.172 and 0.779, respectively).

In addition to the number of spines, we also measured the spine density. The spine density is analyzed depending on its distance to the cell body of pyramidal neuron in CA3 (Picture 3.1, Thorny excrescence is taken as one spine in this analysis). The results show that the spine densities in proximal dendrite and terminal dendrite are not alternated by MS experiences in

early life (Fig 3.10A, p=0.245 and 0.105, respectively), whereas the spine density in medial dendrite shows a tendency towards increase in MS subjects (Fig 3.10A, p=0.097).

3.6 Improved Emotional and Cognitive Behaviours in Stressed Animals

3.6.1 Decreased depressive-like behaviour in forced swim test in stressed animals

Because of a potential litter effect, the number of subjects and the number of litters are pertinent in the behavioural experiments. In the present experiment, 5 litters of control (n=16) and 6 litters of MS (n=19) were tested. Since the differences between the two groups is mostly observed in first several minutes of behavioural testing, most studies only measure parameters for 5 or 6 min in FST, that is 24 hr later after the 15 min habituation test (Porslot et al., 1977; MacQueen et al., 2003; Veenema et al., 2006; Lee et al., 2007). In the present experiment, a 15 min test was conducted and behavioural changes monitored over the time. In addition, the behavioural results are analyzed in two ways: by using data from each individual test subject and by using the average data of each litter, which can test whether there is a litter effect.

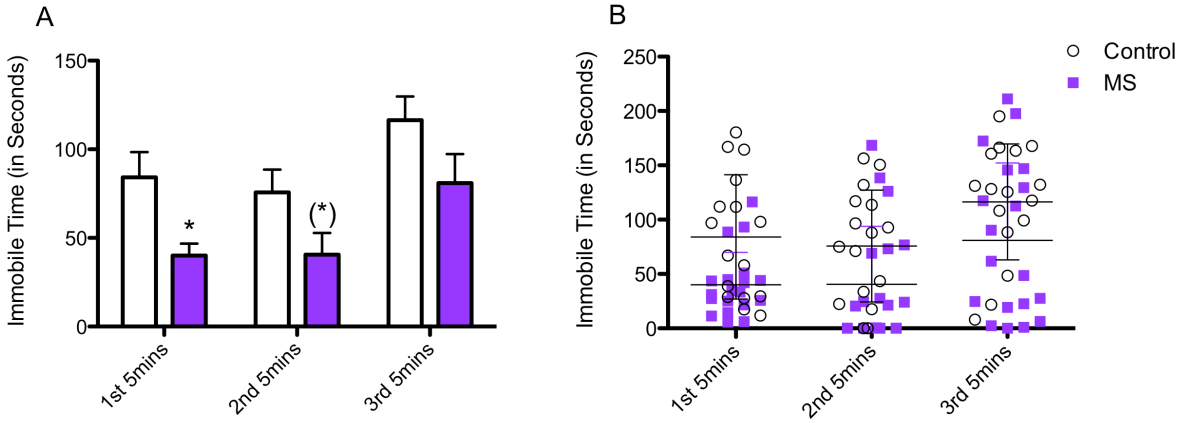


Figure 3.11: Immobility Time for Each Animal in Forced Swimming Test. (A) Immobility time over 15 min: Immobility time is significant reduced in MS during 1st 5 min (p=0.006, n=16 in control, n=19 in MS), and MS results in a trend toward decreased immobility time during the 2nd 5 min (p=0.057). There is no difference in passive behaviour during the last 5 minutes of testing. **(B)** The pattern of immobility time over 15 min for each animal.

MS pups display a significant reduction in immobility time during the 1st 5 min of the test (Fig 3.11A, p=0.006). In the 2nd 5 min of FST, there is a non-significant trend of reduced

immobility in MS mice (p=0.057). In the last 5 min of the 15 min FST, there is no significant difference in time spend immobile between the pups of the MS and the control group.

The pattern of immobility time in each 5 min for each animal can be seen in Fig 3.11B. In the 1st 5 min, it can be seen that the immobility time of control offspring seems expressed more randomly from 11.7 s to 180.3 s, whereas the immobility time of MS offspring is more intensively expressed between 6.1 s and 50.9 s. During the last 10 min of the FST, some MS offspring spend increasing time in immobile pose similar to those in control offspring.

To avoid any effect of litter on FST behaviour, average data for each litter was calculated and analyzed using two-tailed t-test. The pattern of immobility time can be seen in Fig 3.12B. Generally, MS litters show less immobility time in the 1st and the 2nd 5 min. The results are similar to those described above in the analysis of individual data. However, the reduction of immobility time in the 2nd 5 min reaches significance when average data for each litter is analyzed (Fig 3.12A, p=0.045).

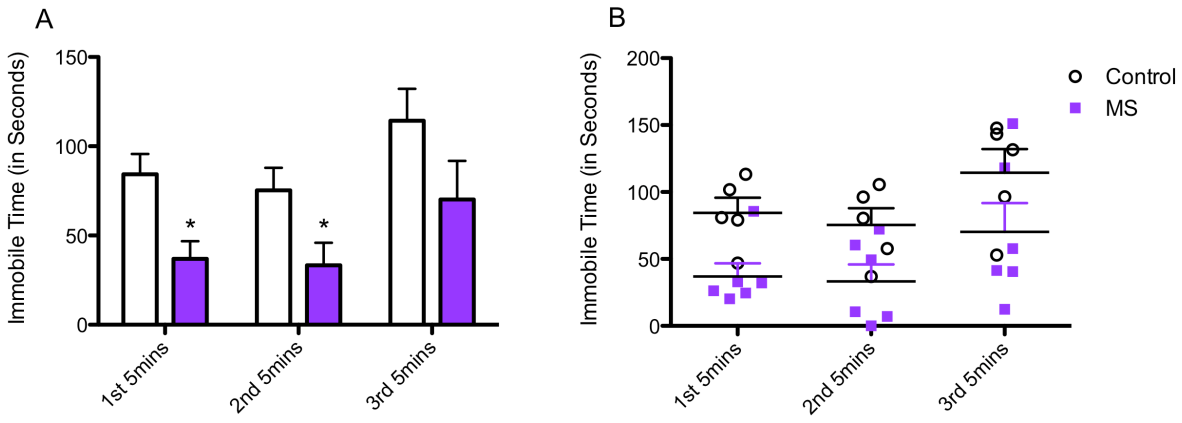


Figure 3.12: Immobility Time for Each Litter. (A) Immobility time over 15 min: The immobility time is significant reduced in MS during the 1st and the 2nd 5 min (p=0.011 and 0.045, respectively; n=5 in control, n=6 in MS), whereas there is no difference during the last 5 minutes (p=0.159). **(B)** The pattern of immobility time over 15 min for each litter.

The FST results indicate that MS in early life has a strong positive influence on emotional behavioural strategies.

3.6.2 Increased body weight and enhanced spatial memory in adulthood of stressed animals

The body weight of 12 animals from each group was measured at 8 weeks (ie. 6 weeks later after MS), and at approximately this age subjects received the spatial learning test. The body weight results show that the MS group is significant heavier than control animals (Fig 3.13A, p=0.043), Although there is only a 5% difference compared to the control group, the increased body weight may indicate that a short period of MS possibly stimulated appetite, and it may be correlated with the elevated emotional response measured in the FST.

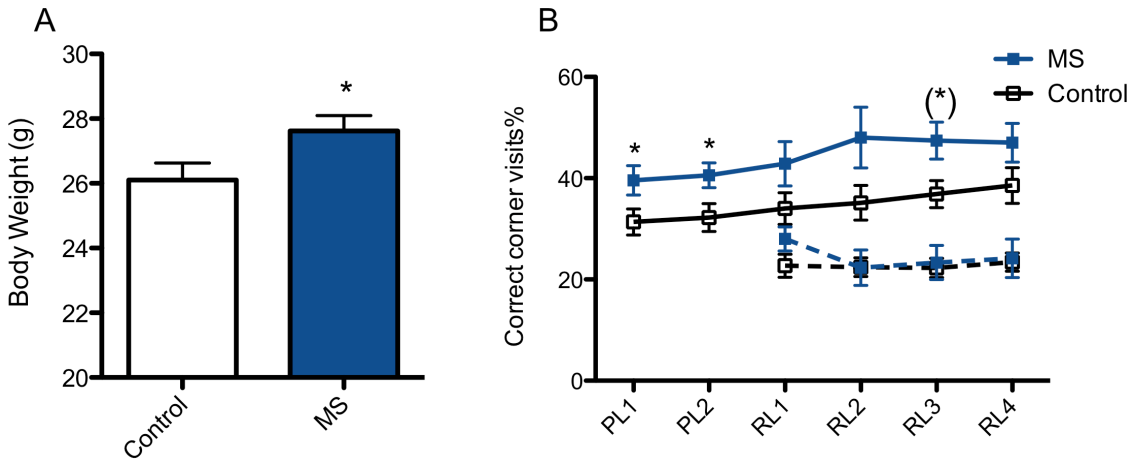


Figure 3.13: Body Weight and Place Learning. (A) Body weight of 8 week old subjects: MS increases the body weight (p=0.043, n=12 per group). **(B)** Improved spatial learning. The percentage of correct corner visits in place learning (PL) is increased in MS group (p=0.044 for PL1 and p=0.031 for PL2, n=14 controls and n=15 MS). In contrast, this increasing ability of spatial learning is not observed in place learning reversal (RL) (repeated measures: p=0.131, n=7 controls and n=6 MS), however, MS mice show a trend towards increase in the percentage of correct corner visits on RL3 (p=0.065). The broken lines show the percentages of visits to a former correct corner in place learning. there is no difference between control and MS mice (repeated measures: p=0.585).

To investigate whether cognition is influenced in the late life by the early life stress, animals were placed in the intelliCage® and allowed to habituate to the new environment. After animals learned to access water by making nosepokes to the door in the corner, 2 days of spatial learning testing was initiated. In this test, water was rewarded in one corner for each animal. The results show that the percentage of visits to the correct corner in MS adult animals is higher than it in control adult animals, either on the first (Fig 3.13B, p=0.044) or the second day (p=0.031) of place learning, which suggests that spatial learning in adulthood is enhanced by experiencing repeated short periods of MS in early life. However, there is no

difference in the percentage of correct responses between control and MS mice in the following 4 days of place learning reversal as demonstrated by a general linear model for repeated measures (Fig 3.13B, p=0.131). Meanwhile, those animals do not show any difference in the percentage of former correct responses (*ie.* rewarded nosepokes in place learning) (Fig 3.13B, p=0.585).

In order to investigate the activity of animals during spatial learning, several parameters were recorded automatically by the Observer software, such as the number of visits at the door (the corner where water is rewarded), the number of nosepokes (required to open the door) and licking behaviour (access to water). The spatial learning test was initiated in late afternoon (16:00). Activity data was collected from 19:00 to 07:00 on the following day during night/dark phase, and data collected from 07:00 to 19:00 is during the day/light phase. Results from one night/day cycle are presented.

There is no doubt that rodents are more activated at night. The present results consistently show (Fig 3.14C) that animals visit the door more frequently during night phase regardless of whether they were stressed in early life (p=6E-08) or not (p=0.046). Both control and MS group perform a greater number of nosepoke and licking (Fig 3.14A, B, p<0.001). The data indicate that MS does not change the subject's nature and they performed more activities during the night/dark phase compared to the day/light phase as controls did.

As presented in Fig 3.13, MS influences cognitive function in the 2-days observation period. Furthermore, cognition may be affected differently during day/light phase and night/dark phase. It can be seen that there is no difference in the percentage of errors made between the day/light and night/dark phases in control subjects (Fig 3.14D, p=0.288), whereas the MS group shows a significantly reduced error rate during the day/light phase (p=0.023). Between the groups, the enhanced spatial learning by MS still can be seen during day phase (p=0.025), however, the improved cognitive function is not evident during the night/dark phase (p=0.272).

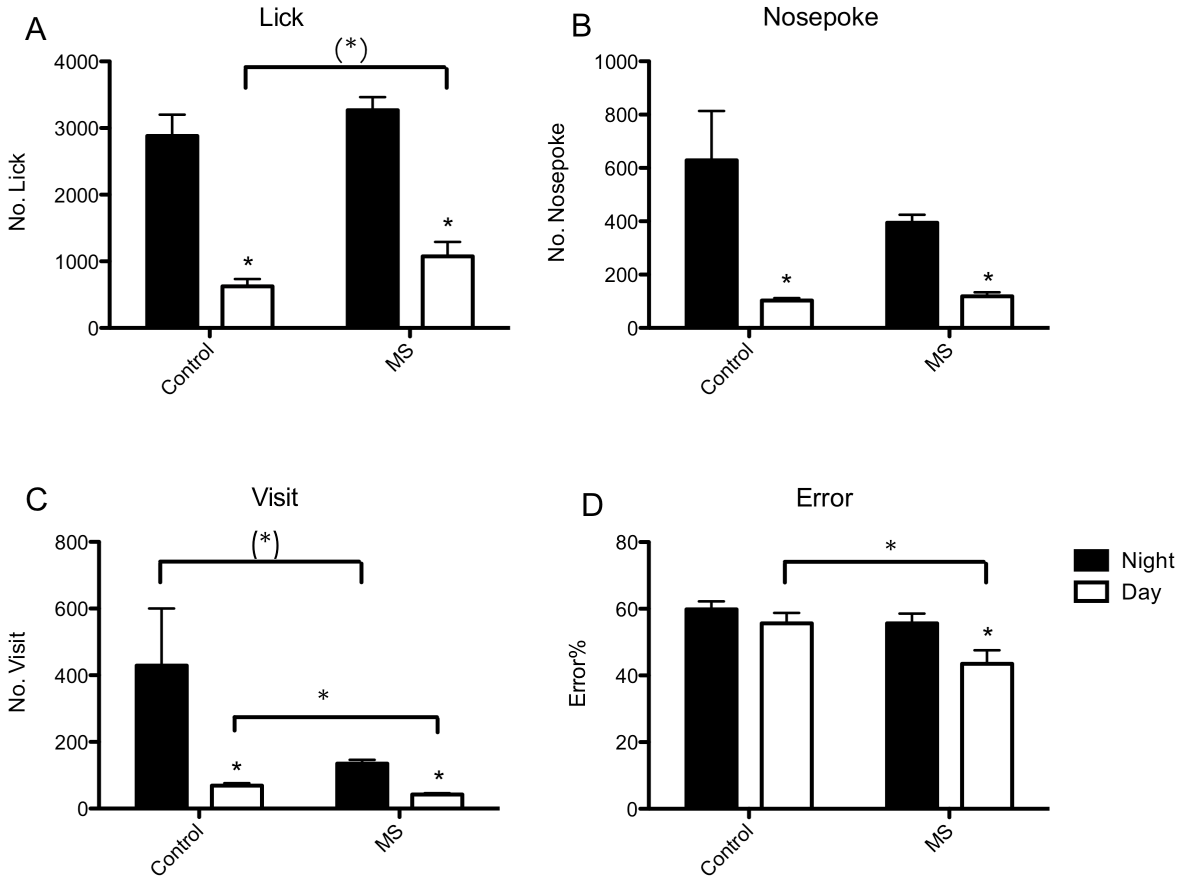


Figure 3.14: Activities during the Day/Light Phase and Night/Dark Phases. (A) Licking behaviour: Both control and MS mice show a significant increase in the number of licks made during the night/dark phase compared to during the day/light phase (p=4.8E-07 and 6.6E-08, respectively). A tendency towards increased licking is observed in MS during the day/light phase (p=0.078), whereas it is not observed during the night/dark phase (p=0.315). **(B)** Number of nosepokes made: A higher number of nosepokes are performed during the night/dark phase in both controls (p=0.009) and MS animals (p=1.23E-08). There is no difference between the groups in day/light phase (p=0.373) or in night/dark phase (p=0.222). **(C)** Number of visits. There is a significant increase in visits during the night/dark phase in controls (p=0.046) and in MS groups (p=6E-08) compared to during the day/light phase, MS mice display significantly fewer visits during the day/light phase compared to controls (p=0.002), whereas only a tendency towards decrease in MS is observed during the night/dark phase (p=0.099). **(D)** The percentage of error: MS animals show reduced errors during the day/light phase (p=0.025) but not during the night/dark phase (p=0.272) compared to controls. Within the groups, there is no difference in error rate between day/light and night/dark phases (p=0.288) in controls, whereas the MS group shows a significantly decreased percentage of error during the day/light phase (p=0.023) compared to the night/dark time. The number of animals is n=14 and 15 in the control and MS groups, respectively.

4. Discussion

The present study is to my knowledge the first to show that ELS, i.e. repeated periods of MS during early childhood, induces a rapid increase of the plasticity-related genes Arc and Egr1 in the mouse hippocampus. Furthermore, it was found that these changes in gene expression are paralleled by histone acetylations, reflected by increased acetylation levels of H3 and H4. Most importantly, the results reveal for the first time a correlation between elevated histone acetylation and increased Arc and Egr1 expression in response to MS. The epigenetic changes observed at the age of PND 16 are paralleled by increased dendritic spine numbers, longer and more complex dendrites on CA3 pyramidal neurons in the MS group at weaning age compared to controls. In contrast to alterations in the hippocampus, the alterations in the PFC were slighter. There was no change in Arc and Egr1 gene expression levels after MS, while only histone H3 acetylation was increased in MS pups. However, it was observed that histone H4 acetylation was increased at promoter regions of Arc and Egr1 genes. At the behavioural levels, improved emotional and coginitive functions were observed late in life in stessed animals. Discussion in details are given piont by point as follows.

4.1 Maternal Separation Stress Model

MS is widely used as an early life stressor. An increase of serum corticosterone observed in the present study clearly indicates an endocrine stress response to this unavoidable stressful situation, and the paradigm of MS applied in the present project is a stressful stimulus for the pups.

However, the paradigms of MS vary across different laboratories. There are two major differences between the MS model employed in the present experiments and other MS models used in other laboratories; namely, the duration of each MS session (i.e. hours/minutes duration per session) and the number of MS sessions (i.e. number of days duration). Usually pups are exposed to MS as either an acute or chronic stressor. Acute stress refers to a single 24-hours separation period in the first week of life; it is also called maternal deprivation. Chronic stress usually involves 1 - 3 hours of daily separation over 12 - 21 consecutive days during the first 3 weeks after birth (Stanton et al., 1988; van Oers et al., 1998; Wigger and Neumann 1999; George et al., 2010; Schmidt et al., 2011; Tata 2012). In the present experiments, 3 hours daily MS for 3 consecutive days paradigm is somewhat ambiguous in terms of definition as acute or chronic. Behaviourally, it is believed that chronic exposure to stressful stimuli induces anhedonia-like behaviour, whereas acute stress rarely results in this reward-related behaviour (For a review, see Sun et al., 2012). Based on this principle,

although our paradigm was conducted over 3 consecutive days versus the commonly-used 24 hour acute stress, our MS paradigm can likely be considered an acute stress as 3 days of MS did not result in any difference in sucrose preference test results compared to control pups (data not shown), but differences in depressive like behaviour and spatial learning were observed.

The second major difference between this and commonly used MS paradigms is the time of life at which pups were exposed to the stressor (i.e. age of subjects). We applied MS after the SHRP of the HPA axis (i.e. after PND 14 for rodent), whereas others were conducted during the SHRP or across this period. A previous study in this laboratory investigated the stress effects on dendritic structure at 3 time windows depended on the SHRP: prior to the SHRP, during the SHRP and after the SHRP, respectively (Bock et al., 2005a). It was observed that there were different effects induced by MS applied in different time windows. Hence, it is reasonable to reconsider the effects of MS paradigms, which are conducted across the SHRP because there were different effects if MS applied in different time windows. Evidence from the previous study shows that MS applied after the SHRP increased spine density and dendritic length in the cingulate cortex, which is opposite to the effect observed prior to the SHRP or in other MS models (Bock et al., 2005a). We assumed that the period immediately after the SHRP is early childhood for mouse, and the pups are at a time point when they start to open eyes and actively explore their environment. Accordingly, isolated novel space is possibly like a novel environment for our pups to explore. Although there is no direct evidence to indicate that a novel environment improves cognitive behaviour, many studies have found that an enriched environment (to explore) prevents cognitive impairment and/or improves cognitive behaviour (Lee et al., 2012; Simão et al., 2012; Xie et al., 2012; Yao et al., 2012). It is possible that the behaviour of exploration is important for development of the brain. Thus, in our MS paradigm, it is assumed that taking MS pups away from homecage provides these pups a novel and/or enriched environment, and allows them to explore a novel environment may result in positive effects on emotional and cognitive behaviours.

In addition, research reported that maternal care can reduce stress responsiveness (For a review, see Walker, 2010; Aniko and Baram, 2011), and evidence also shows that the separated dams performed more maternal behaviour including increased time spending on nest, increased licking and grooming after reunion (Millstein and Holmes, 2007; Zimmerberg and Sageser, 2011). Although maternal behaviours were not investigated in the present study,

it is possible that there was an increase of maternal care after reunion, which reduced MS pups stress responsiveness and enhanced emotional and cognitive functions as observed.

4.2 Enhanced Neuronal Plasticity-Related Gene Expression

Arc protein synthesis plays a crucial role in learning-related synaptic plasticity and memory function, and it may be related to the structural changes that are observed after early stress exposure (Bock et al., 2005b, Tzingounis and Nicoll, 2006; Alme et al., 2007; Huang et al., 2007). At mRNA level, an increased expression of Arc in the hippocampus was observed in stressed animals, which is consistent with other studies that a brief exploration of novel environment induced Arc gene expression in hippocamupal and parietal cortex neurons (Guzowski et al., 1999; Vazdarjanova et al., 2002). In addition, evidence shows that gene expression of Arc occurrs rapidly and transiently after exploration (Guzowski et al., 1999). Later on, it was confirmed that either Arc mRNA or Arc protein are coordinately induced in populations of neurons in the hippocampus by spatial exploration (Ramírez-Amaya et al., 2005). However, some studies of adult rats observed that stress exposure increased Arc mRNA levels in the PFC, but not in the hippocampus and the parietal cortex (Mikkelsen and Larsen, 2006; Schiltz et al., 2007). This region-specific pattern is inconsistent with the present findings that increased Arc mRNA was only observed in the hippocampus and not in the PFC. There are three main reasons can be considered. Firstly, the increased Arc mRNA expression observed in above studies was quantified in the mPFC of the brain sections, whereas homogenization of whole PFC was used in our study. It is possible that subregion-specific subtle changes in the mPFC were masked due to the analysis of the whole PFC. Secondly, different stressors may contribute to different results. Indeed, at least, a study has revealed that different stressors induce different degrees of activation in the brain (Cullinan et al., 1995). It was reported that a higher c-fos expression was induced in the brain after 30 min forced swim compared to those exposed to 30 min restraint stress, which may suggest stressor that includes spatial exploration has a greater effect on the brain. In our stressed models, as was discussed in chapter 4.1, MS after the SHRP may include spatial exploration, which may explain the increased Arc mRNA induced in the hippocampus of MS pups. The third reason is GR expression levels in different brain regions may influence responsiveness of these regions to stress events. Evidence shows that GRs are widely expressed in the hippocampus (Sánchez et al. 2000), and it has been demonstrated that reduction of GR protein is accompanied by a decrease of Arc protein in the hippocampus of $GR^{(+/-)}$ mice (Molteni et al. 2010). In line with this finding, a recent study showed that Arc expression in the hippocampus can be activated via GR's and the CaMK (Ca^{2+}/Calmodulin-dependent protein kinase) IIα -BDNF-CREB

(cAMP response element-binding) pathway, and the mechanisms are critically involved in the regulating of memory consolidation (Chen et al., 2012). Therefore, it is speculated that the elevated corticosterone induced by MS may activate GR in the hippocampus, further increases Arc gene expression.

Egr1 is another target gene in the present project. It is one of the best-characterized activity-regulated immediate early genes for their roles in synaptic plasticity and memory functions. Egr1 encodes a nuclear transcription factor that is rapidly induced in specific brain regions during memory consolidation and recall (For a review, see Davis et al. 2003; Knapska and Kaczmarek 2004). It has also been shown that Egr1 is required for the expression of long-term memory (Jones et al. 2001; Bozon et al. 2003; Davis et al. 2010). The result of Egr1 gene expression in the present study was similar to Arc gene expression, with elevated Egr1 observed in the hippocampus. This result is consistent with our previous studies, which observed an elevated level of Egr1 gene transcription in the forebrain following an acoustic imprinting stimulation in early life (Thode et al., 2005). Egr1, as a transcriptional regulatory protein, is involved in regulating other gene expression and its gene expression is commonly used as an indicator for neuronal activation. There are many reports indicating that learning, stress and early-life experiences can induce alterations of Egr1 expression (Meaney et al., 2000; For a review, see Knapska and Kaczmarek, 2004). One rodent study investigated the time-course of immediate early genes expression after acute stress. In the respective study, the highest Egr1 mRNA expression levels were observed both in the PFC and in the hippocampus at 30-60 min after exposure to acute forced swim stress, and this elevation was returned back to basal levels within 120 min (Cullinan et al., 1995). In the present study, similar time-course pattern of Egr1 mRNA expression after MS was observed in the hippocampus, but not in the PFC. It could be explained by technical and procedural differences as discussed above regarding Arc mRNA. In brief, whole PFC was used instead of using subregions of PFC to detect Egr1 mRNA levels, different stressor employed and widely expressions of GR in the hippocampus could contribute to profound gene expressions occurring in the hippocampus but not in the PFC. Refering to the association between GR and Egr1, evidence shows that stress-induced activation of GR results in Egr1 expression (Revest, et al., 2005).

In summary, the brief MS used in the present study may be considered as a relatively “low dose” stressor, which includes exposure to a novel environment where pups can explore. The increase in expression of plasticity related genes is not only triggered by separation stress, but also related to exploration of a novel environment. In addition, although there were no

changes in gene expression in the PFC, one cannot exclude some modest alternations may occur in subregions of the PFC that were not detectable in whole PFC homogenization. It is also worth noting that gene expression levels were only detected at three different time points in 2 hours after the last session of MS, while alterations may occur in the PFC but not in these 2 hours.

4.3 Region-specific Enhanced Histone Acetylations

Histone acetylations are believed to facilitate gene transcription, and histone H3 and H4 have more residues for modifications compared to hitstone H1 and H2. It is believed that acetylations of histone H3 and H4 play a dominant role in chromatin remodelling (for a review, see Kouzarides, 2007). The results of my study showed that MS resulted in elevated acetylations on histone H3 in the PFC, and both acetylated histone H3 and H4 were increased in the hippocampus. This is reflected in the pattern observed in gene expression and indicates that the hippocampus was more sensitive to MS showing more profound MS-induced alternations than the PFC.

It was previously observed that elevated histone H3 acetylation was induced in the hippocampus after fear conditioning (Levenson et al., 2004), and repeated social defeat resulted in an increase of histone H3 acetylation in the hippocampus, whereas there was no alterations of histone H4 acetylation in any other brain region (Hollis et al., 2010). Moreover, one study found that an increase of histone H3 acetylation induced by repeated social defeat was only seen in one subregion of the PFC: the infralimbic mPFC (Hinwood et al., 2011). These observations of increased histone H3 acetylations are consistent with the findings in the present study. Although no alteration of histone H4 acetylation was observed in the brain of these stressed models, a study reported that 2 hours exploration in the experiment chamber prior to fear conditioning increases hippocampal histone H4 acetylation, but not histone H3 acetylation (Levenson et al., 2004). This may suggest that histone H4 acetylation is related to spatial exploration, which is consistent with the present findings that elevated acetylation of histone H4 was induced by MS (containing a spatial exploration as discussed in chapter 4.1). A recent study in MS models consistently shows an elevation of histone H4 acetylation in the forebrain (Levine et al., 2012). At behavioural levels, mounting evidence consistently shows that enhanced histone acetylations via inhibition of HDACs alleviate cognitive functions (Guan et al., 2009; Peleg et al., 2010; Gräff et al., 2012). As opposed to adult stress (Hinwood et al., 2011; Castellano et al., 2012; Hollis et al., 2012) and DNA metylations (Murgatroyd et

al., 2009; Frankin et al., 2010), only a few studies have identified changes in histone acetylation after early life stress (Levine et al., 2012).

Evidence for a direct correlation between the increase of stress hormones and enhanced histone acetylation comes from a study of Roozendaal and colleagues, where it was shown that the systemic application of corticosterone increases histone acetylation in the insular cortex and the hippocampus (Roozendaal et al., 2010). Molecular studies may suggest some possible pathways of corticosterone regulating histone acetylations. It is well-known that corticosterone facilitates Ca^{2+} conductance, and CaMK IV can phosphorylate CREB binding protein (CBP), which exerts HAT activity (Impey et al. 2002). This indicates that elevated corticosterone after MS may increase Ca^{2+} influx and activates CaMKs, subsequently enhances HAT activity of CBP, which facilitates histone acetylations and in turn upregulates CREB-regulated gene transcriptions. For instance, Egr1 is one of CREB target genes (Fass et al., 2013). Although class II HDACs are also phosphorylated by CaMKs (McKinsey et al., 2001), Ca^{2+} influx can induce nuclear export of HDAC5, one of class II HDACs, in hippocampal neurons (Chawla et al., 2003). Thus, it is assumed that in the present study elevated corticosterone induced by MS increases histone acetylation levels via activating HAT in nucleus. This leads to increase in histone acetylations. via nuclear export of HDAC, which leads to decrease in histone de-acetylations. In this pathway, activation of CaMKs is an important factor. A preliminary study in our lab using gene arrays indicates that gene expression level of CaMK 2g is increased in the hippocampus after MS (data not shown).

An important synaptic plasticity factor, the N-methyl-D-aspartate (NMDA) receptor, may be involved in another pathway of corticosterone regulating histone acetylations. An investigation in the hippocampal CA1 region demonstrated that activation of the NMDA receptor increased histone H3 acetylation (Levenson et al., 2004). However, does activation of NMDA receptor occur as a response to stress or stress hormones in early life? Some studies have demonstrated that MS or stress hormones upregulates NMDA receptors (Sircar et al., 2001; Lee et al., 2003; Owen and Matthews 2007; Ryan et al., 2009). A preliminary study in our lab using gene arrays indicates that gene expression level of a subunit of NMDA receptor, Grin2d, is increased in the hippocampus of MS (data not shown). Specifically, a study found that chronic treatment with a high dose of corticosterone increased expression of the NMDA receptors NR2A and NR2B mRNA in the dentate gyrus and CA3 respectively before puberty, but not after puberty (Lee et al., 2003). This pattern of age-dependent effect is similar with it in a study of corticosterone administration (Oda and Huttenlocher, 1974). Furthermore, in

downstream of this pathway, it has been found that increase of histone H3 acetylation induced by activation of NMDA receptor is through either cAMP-protein kinase C- (PKC) or extracellular signal-regulated kinase (ERK)- cAMP-protein kinase A- (PKA) (Levenson et al., 2004, and PKA is known to phosphorylate CREB, which in turn mediates HAT activity (Yuan and Gambee, 2001; For a review, see Carlezon et al., 2005). However, although histone H4 acetylation was increased by 2 hours exploration in chamber prior to a fear conditioning experiment, it was independent of activation of ERK2. In addition, in vitro results confirmed that histone H4 acetylation is regulated neither by PKA nor PKC (Levenson et al., 2004). This may suggest that activation of NMDA receptor via ERK-PKA/PKC is possibly specifically involved in regulation of histone H3 acetylation, but not histone H4 acetylation.

All in all, our finding of ~ 2.5 fold increased of serum corticosterone after MS may not casually increase histone acetylations. Corticosterone may elevate of histone H3 and H4 acetylation through both pathway of CaMKs and pathway of NMDA-ERK-PKA/PKC, while the latter one may regulate acetylations more specifically on histone H3.

4.4 The Association between Histone Acetylations and Target Gene Expressions

The present results demonstrated that repeated short periods of MS during early life lead to a rapid increase of the plasticity-related genes Arc and Egr1 expression in the hippocampus. Moreover, we were able to show that these rapid stress-induced changes are paralleled by increased acetylation levels of histone H3 and H4. Most importantly, the present results reveal for the first time a direct correlation of histone acetylation with increases in plasticity-related gene expression directly after stress exposure. Enhanced acetylation of histone H4 was found to be associated to the promoter regions of Arc and Egr1, whereas the enhanced acetylation of histone H3 showed less profound association as little effect was seen in the PFC and no effect in the hippocampus. It is assumed that acetylation of histone H4 plays a dominant role in activation of Arc and Egr1 gene transcription compared to acetylation of histone H3. Thus, although there is no significant global increase in acetylation of histone H4 in the PFC, an increased acetylation of histone H4 specifically associated with the activation of Arc and Egr1 gene was shown after MS.

The elevations of gene expressions are most likely mediated by stress-induced histone acetylations, since the levels of acetylated histone H3 and of H4 were increased in MS animals and ChIP-qPCR analysis indicates an association between histone acetylation and

gene expression, that is the increase in acetylation of histone H4 activates Arc and Egr1 gene transcriptions in the hippocampus, while increased Arc and Egr1 gene expression levels were observed in this region. It is important and inspiring to note that these events reflect very rapid epigenetic alterations in response to an environmental challenge: the association between histone H4 acetylation and the promoter region of Egr1 was observed immediately (0 min) after the last session of MS, and Egr1 gene expression levels were consistently observed immediately after MS in the hippocampus. However, in term of Arc, the association between histone H4 acetylation and the promoter region of Arc was not observed immediately after MS, whereas increased Arc gene expression levels were shown at this time point (0 min). It is assumed that the immediate increase in Arc gene expression may be caused by an accumulating effect as MS pups had been exposed to stress for 3 hours/day in consecutive 3 days. Thereby, although increased histone H4 acetylation did not occur at the promoter region of Arc immediately at the end of the last 3 hours-stress exposure, there may be an accumulating effect regulates Arc gene expression during the 3 sessions of 3 hours MS. Indeed, a study identified Arc as a direct target of Egr1 and it has shown that Egr1 can bind the Arc promoter (Li et al. 2005). Egr1 protein is possibley accumulated during the 3 sessions of MS, and activate Arc gene transcription, in turn increase Arc gene expression levels.

A recent study of a novel HDAC inhibitor found that inhibition of HDACs induced increased histone H3 and H4 acetylations, and an enhanced expression of CREB target gene Egr1 (Fass et al., 2013), which supports the present result that Egr1 is correlated with histone acetylation. Moreover, enhanced histone acetylation also shows an association with Egr1 binding to promoter region of GR. Evidence demonstrates that increase of histone H3 acetylation induced an increase of Egr1 binding to neuron-specific exon 1_7 GR promoter (Nr3c1) and a decrease of DNA methylation at Nr3c1 (Weaver et al., 2004). Hence, it is speculated that the elevation of histone H3 acetylation that were observed in our MS animals may also increase Egr1 binding to Nr3c1 and DNA de-methylation at Nr3c1, subsequently enhance expression of GR protein and GR signalling. It is known that corticosterone binding to GR recruits a chromatin remodeling complex in the nucleus, which induces HAT activity and increases histone acetylation levels (For a review, see Schoneveld et al., 2004). Thereby, under the long-term/chronic stress, the cycle: stress-activated GR signalling - histone acetylation - Egr1 binding to de-methylated GR promoter - enhanced GR expression and signalling, may be continuously stimulated by chronic stress, resulting in dysfuctional GR signalling, and eventual stress-related diseases symptomatology.

4.5 Increased Apical Dendrite Complexity and Spine Number in Hippocampal CA3 Neurons of Stressed Animals

As mentioned in introduction, Arc and Egr1 are neuroplasticity-related genes, and it has been suggested that these two genes are correlated with the synaptic structural changes. Particularly, it has been found that Arc mRNA is translocated to dendrites and accumulates at active synaptic sites, where it is locally translated (Lyford et al., 1995). The present study observed increased expression of Arc and Egr1 mRNA levels in the hippocampus, but no changes in the PFC. In line with this interpretation we investigated changes in neuronal structure in the hippocampus in response to early life stress. It was found that MS increased spine numbers in medial and terminal dendrites and increased the complexity of apical dendrites of CA3 pyramidal neurons. Taken together with our previous study (Bock et al., 2005b), it may suggested that dendritic structure changes are possibly correlated with Arc expression. In addition, a study reported that a significantly increased number of large spines, which were marked with Arc were found in CA1 neurons immediately after exposing mice to a novel environment for 60 min, whereas no similar change was observed when mice were exposured for 15 min to a novel environment followed by return to homecage for 45 min (Kitanishi et al., 2009). It has been suggested that duration of stressor exposure may be important for development of spines, and a brief (15 min) novel exploration is probably insufficient to result in Arc expression and dendritic structural changes. It is also possible that Arc-positive spines occur rapidly and transiently, and stress-induced spines were faded 45 min after return to homecage. In the present study, consecutive 3-day MS may speed up development of dendritic structures including significantly increased complexity in apical dendrites of CA3 and increased spine number in medial and terminal dendrites. However, the spine density was not increased as we observed in the mPFC in the previous study (Bock et al., 2005a), and only a tendency toward increase was observed in medial dendrites of pyramidal neurons. It is necessary to emphasize that we detected morphological changes 5 days later after the last session of MS in order to allow MS exert effect on the development of dendritic structure. Evidence suggests that dendritic spines of pyramidal neuron can increase rapidly, 24 hr after stimulation in some cases (Leuner et al., 2003; Holtmaat et al., 2006), and the growth and retraction of spines remained a dynamic balance under basal conditions (Holtmaat et al., 2005). It is assumed that any transiently increased spines may be faded and the growth and retraction of spine possibly returned to a dynamic balance after 5 days in the present study, however, stress-induced increase in complexity and length of dendrites remained, these result in no alterations in density of spines but an increased number of spines observed as there was an longer dendrite in CA3 of MS animals. Interestingly, this dynamic

balance of spine growth is affected by the animals' age. Under basal conditions, a in vivo study found that the number of pyramidal neuron spines in mice which disappeared exceeded the number of spines which appeared during PND 16-25, whereas this loss was not observed after PND 25 (Holtmaat et al., 2005). In the present study, dendritic structure examination was carried out on PND 21, which is during PND 16-25 when disappeared spines exceeds appeared ones in pyramidal neurons. However, it is unclear whether and how MS influences the balance of spine growth. The present results may suggest that MS possibly stabilizes spines and increase number of spine during separation on PND 14-16.

Recent studies using rat hippocampal slices suggest that corticosterone facilitates growth of dendritic spines via GR signalling (Jafari et al., 2012; Komatsuzaki et al., 2012). These results are consistent with the present study that dendrite growth may be facilated by MS-induced release of corticosterone. However, in rats at age of approximal 50 days, some studies of chronic corticosterone administration reported that excess glucocorticoids induces repressive dendritic structure in CA3 pyramidal neurons (Woolley et al., 1990), and redistribution of apical dendrites in the mPFC reflected by an increased number of intersections in proximal dendrites and a decreased number of intersections in medial and terminal dendrites (Wellman, 2001). This indicates the effect of corticosterone on dendrite is possibly age-dependent. Indeed, almost 40 years ago, a pharmacological study has demonstrated that corticosteroid administration at PND 9 facilitated dendrite growth in 3-week old rats and repressed growth at 6-week old (Oda and Huttenlocher, 1974).

In addition to age-dependent effect, effect of corticosterone on dendrites also shows a region- or neuron-specific alteration. The atrophy in the hippocampus is specifically found in the apical dendrites of pyramidal neurons in CA3 but not in pyramidal neurons in CA1 or in granule neurons in dentate gyrus (Woolley et al., 1990). In the present study, the increased complexity and apical dendrites were also observed in pyramidal neurons of CA3, but not in granular neurons in dentate gyrus (data not shown). The possible explanation is corticosterone facilitates Ca^{2+} conductance in hippocampal pyramidal neurons, whereas CA3 pyramidal neurons contain low levels of Ca^{2+} binding proteins and are more vulnerable to high levels of intracellular Ca^{2+} (Woolley et al., 1990). However, as described in introduction, stress-animals show an adaptation process in some cases. Evidence shows that prenatal and postnatal repeated stress enhances expression of CaMK II and Ca^{2+} channels in the hippocampus, specifically in CA3 (Cai et al., 2011), which could be a compensative regulation in response to an increase of intracellular Ca^{2}. An increase in thickness of

postsynaptic density and a decrease in width of synaptic cleft were also observed in the CA3 under electron microscopy (Sun et al., 2006), while these changes are accompanied by improved learning and memory (Sun et al., 2006; Cai et al., 2011). Coincidentally, alteration of CaMK II expression also shows an age-dependent pattern as that observed in corticosterone-induced alterations of dendritic structure (Oda and Huttenlocher, 1974), which refers to prenatal stress-induced the compensation was only observed in young rats (PND 30) (Cai et al., 2011), there was no change in adult rats (PND 80) (Fumagalli et al., 2009). Thus, it is possible that the machinery of dendritic structure alteration in our stressed mice is dependent on both age-and region-/or neuron-specific effects. Taken together, it is assumed that MS in early life induces release of corticosterone, which increases intracellular Ca^{2+} and facilitates Ca^{2+} -denpendent phosphorylation of CaMKs (may include the compensative increase of CaMKs) in CA3, in turn resulting in autophosphorylation (Ca^{2+} -indenpendent) of CaMKs. Subsequently, the activated CaMKs phosphorylate HATs and HDACs, while HDACs can be exported from the nucleus by Ca^{2+} influx (discussed in Chapter 4.3), these lead to enhance histone acetylations. Further, the increased histone acetylations at the promoter regions of Arc and Egr1 activate Arc and Egr1 gene expression. Arc and Egr1 are well-studied as synaptic plasticity genes. Studies reported that stress induces Arc and Egr1 expressions, and these expressions are involved in dendritic structure remodeling and synaptic plasticity (Cole et al., 1989; Wisden et al., 1990; For a review, see Yuste and Bonhoeffer, 2001; Rodríguez et al., 2005; Bramham et al., 2008). In context to the above discussion, it is assumed stress-induced Arc and Egr1 expressions via histone acetylations eventually induce enlargenment of apical dendritic stucture in CA3 of stressed animals at PND 21.

Moreover, a recent study supports the association between CaMKs and Arc in our assumption, authors reported that a lack of CaMK IIß resulted in a loss of Arc accumulation in dendritic spines (Okuno et al., 2012). Another recent study consistently found that GR activates Arc expression in the hippocampus via activation of CaMKIIα-BDNF-CREB pathway, and this activation mediates memory consolidation (Chen et al, 2012). Refering to Eg1, although some reviews have summarised the essential role of Egr1 in learning and memory, as well as in neuronal plasticity (Bozon et al., 2003; Davis et al., 2003), there are very limited studies to suggest that molecular mechanisms of Egr1 regulating dendritic structure in stressed models. A study reported that an increase of hippocampal CREB mRNA was observed in chronic stressed rats (Sun et al., 2006), which can regulate histone acetylations via binding to CBP (one of HATs). Also, increased thickness of postsynaptic density was observed in CA3 accompanied by enhanced CREB expression (Sun et al., 2006).

It is assumed that the transcription of the CREB target gene, Egr1, may be subsequently upregulated by increase of CREB and correlated with spine structure changes.

Although for our data a direct causality between the histone acetylations and neuronal structural changes has not yet been shown, there is evidence from some recent pharmacological studies which revealed that the inhibition of HDACs induces changes of pre- (synapsin-1, synaptophysin) and postsynaptic (dendritic spines) structures and sprouting of dendrites (Fischer et al., 2007; Calfa et al., 2012; Ricobaraza et al., 2012; Fass et al., 2013). Another study reported that spine density is significantly higher in the CA1 and dentate gyrus regions of HDAC2 KO mice. Conversely, spine density is reduced in HDAC2 overexpression mice (Guan et al., 2009).

In summary, taken together with other studies using administration of corticosterone and inhibition of HDADs, MS-induced molecular and cellular alterations observed in the present study are tempting to speculate that stress-induced enhancement of neuronal structure may be mediated by increased Arc and Egr1 gene expressions via corticosterone-induced histone acetylation.

4.6 Behavioural Manifestation

Although chronic MS is used to induce anxiety-like and depressive-like behaviours, accumulating evidence suggests that MS during the early life may also improve emotional and cognitive behaviours later in life, depending on the stressor type, duration and age at exposure (see chapter 1.2.3). For instance, recently, it was found that MS enhanced object location memory and improved avoidance learning in rodents compared to non-stressed control (Abraham and Gruss, 2010; Makena et al., 2012). This is consistent with the present behavioural findings that MS reduced immobility time in forced swim test (decreased depressive-like behaviour) and increased percentage of correct corner visit in spatial learning test (improved spatial memory). In addition, we observed that MS pups made fewer visits to all corners of intellicage, but MS animals showed an increased number of lick (in condition of subject choosing correct corner and makes nosepoke), which also indicates MS-enhanced spatial memory since MS mice spent less time trying (visit) to access their needs (lick). Interestingly, from the data that was collected in the first 24 hours of the experiment, it was seen that spatial learning was only enhanced during the day/light phase but not during night/dark phase. Since light takes a role of mild stressful stimuli for rodents, it is assumed that MS subjects possibly perform better in coping with stress compared to controls in later

life. Consistently, some studies using different behavioural tests provided evidences that MS or prenatal stress can exert positive effects on emotional development and cognitive competence (Sanders and Anticevic, 2007; Schäble et al., 2007; Schroeder et al., 2012).

At the behavioural level, the hippocampus has been considered as an integrator of emotion and cognition (For a review, see Femenía et al., 2012), which is connected with other regions of brain, such as PFC. Enhanced spatial learning specifically correlates with the critical role of the hippocampal CA3 region in encoding of new spatial memory (For a review, see Kesner, 2007). Thus, the improved spatial learning that were observed in our MS animals may result from stress-induced enlargement of apical dendritic trees and increased spine numbers in pyramidal neurons of CA3. Recently, it was reported that chronic MS during the early life produces a trend towards decreased apical dendrite length in the CA3, which may be relevant to the impairment of hippocampal-dependent memory (Eiland and McEwen, 2012). This trend of decline in apical dendrites becomes significant and anxiety-like behaviour was induced after additional chronic stress in adulthood (Eiland and McEwen, 2012). From the aspect of stress effects, these results are inconsistent with the present results because of different stress paradigms. However, it consistently demonstrates the correlation between dendritic structure in the CA3 and emotinal and cognitive behaviours as observed in the present study.

In the discussion of molecular and cellular results, it is known that activation of CREB takes an important role in histone acetylations and Arc and Egr1 gene expressions, which may correlate to structural alterations in CA3. Moreover, at the behavioural level, evidence consistently shows that activation of CREB in the hippocampus exerts anti-depressive effect (For a review, see Carlezon et al., 2005).

Since cortiosterone is assumed to regulate histone acetylations and other subsequent alterations in the present study GR, as one type of corticosterone receptor, its signaling results in histone aceylations, we cannot ingnore the importance of GR in behavioural manifestation. In GR knockout and $GR^{(+/-)}$ mouse models, a low expression of GR was observed, and these $GR^{(+/-)}$ mice show greater depressive-like behaviours after stress exposure compared to controls (Boyle et al., 2005; Ridder et al., 2005). In consistent, the present study observed an elevation of corticosterone, which is supposed to activate GR, further results in less depressive-like behaviour as observed. However, other studies show behavioural

consequences of GR over expression are conflicting (Wei et al., 2004, 2012b; Ridder et al., 2005).

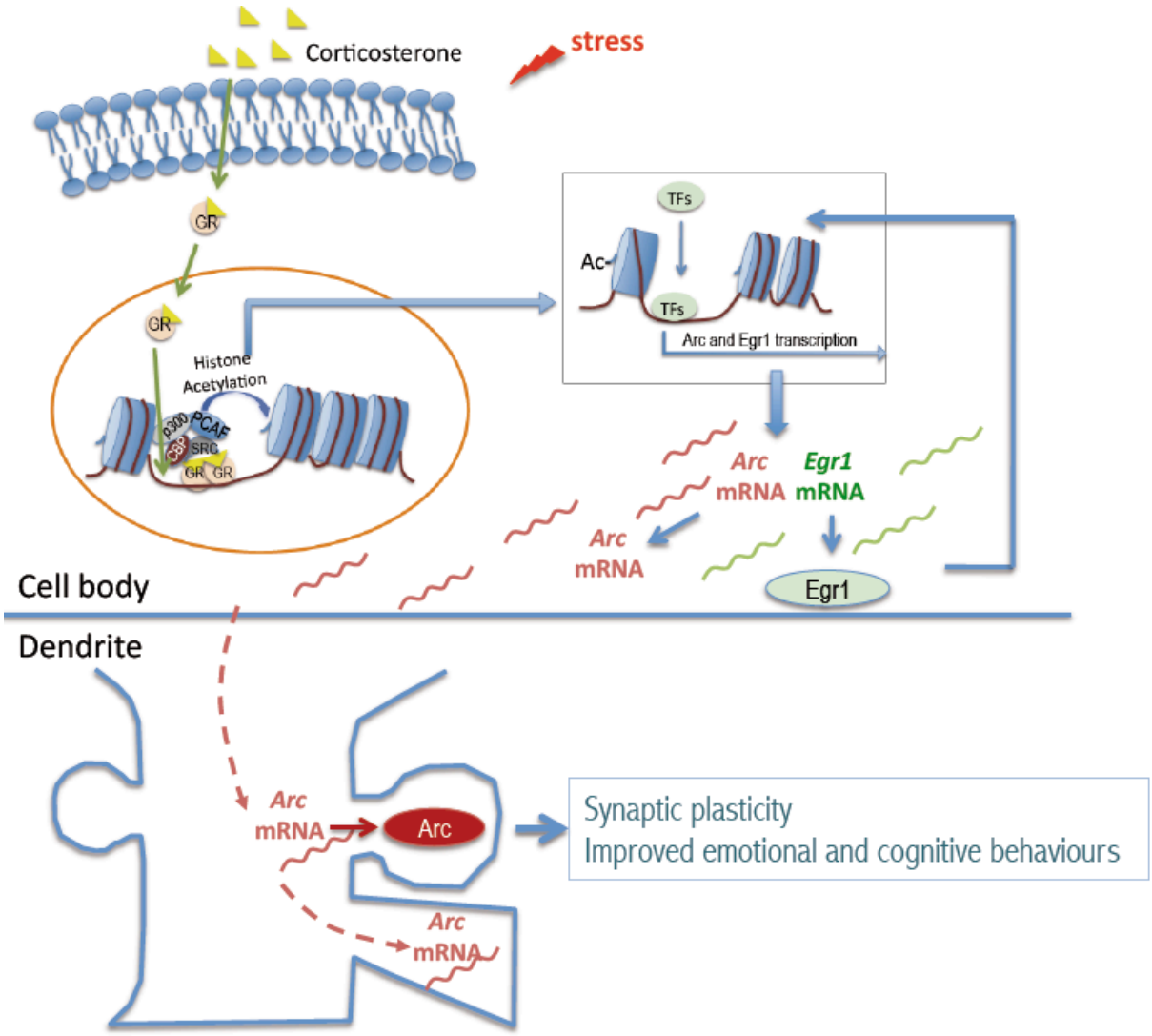


Figure 4.1: The Illustration of the Model of Epigenetics Invloved in Dendritic Structure Alternation Induced by Maternal Separation. The model states that the stress of maternal separation increases the release of corticosterone in pups, which further increases its binding to GR. Activation of GR signaling conducts the stimulus to nucleus and recruits CBP complex, which can exert the HAT functions and increase histone acetylations. The acetylated histone is negatively charged and repels DNA, which also holds negative charges. Thus, the opened chromatin allows transcriptional factors (TFs) bind to promoter region of Arc and Egr1, and activate gene transcriptions. Further, increase Arc and Egr1 gene expressions. Egr1 protein is translated and transported to nucleus, where it facilitates other gene transcription as Egr1 itself is a TF. Arc mRNA is carried along microtubules and translocated to the dendritic spine and then translated locally, which is possibly correlated with the alternation of dendritic structure followed by behavioural changes late in life (the underlined parts in text were comfirmed in the present study).

In conclusion, the most profound alterations induced by MS were observed in the hippocampus. It was demonstrated for the first time that brief separation stress in early childhood up-regulates histone acetylation around the promoter regions of the synaptic plasticity genes Arc and Egr1. We assume that the stress-induced histone modifications together with the regulation of plasticity-related genes interfere with the establishment and "formatting" of hippocampal circuits. Thereby inducing long-lasting structural changes in hippocampal synaptic connectivity, and thus determining the emotional and cognitive potential of this brain region later in life in order to better cope with environmental challenges (see Figure 4.1).

5. Outlook

The present project focused on histone acetylations in the hippocampus and the PFC induced by early life stress, it also concentrated on the possible connections between these immediately epigenetic changes and later dendritic structures alterations, as well as long-term behavioural consequences. An increasing number of studies indicate that corticosterone may regulate epigenetic changes, which are correlated with altere gene expression, dendritic morphology and behavioural consequences. However, there is no direct evidence to reveal how corticosterone triggers epigenetic mechanisms underlying different phenotypes at different levels. As it was discussed above, it is assumed that corticosterone regulates the stress-responses, including epigenetic alternations, and subsequently induced morphological alternations and behavioural changes. Thus, in order to further investigate the assumptions presented, the following pharmacology experiments may be conducted:

(1) *Corticosterone and/or GR antagonist administration*. The administration of corticosterone during the same time-windows as MS was conducted. Following this, histone acetylation levels, morphological and behavioural changes should be examined to confirm whether all alternations observed in the present project were induced by elevated corticosterone during specific time-windows. Or GR antagonist can be applied to reverse the effects that were observed in the present study. To conduct this experiment, the route of administration is critical; forced-feeding, subcutaneous, intraperitoneal and other injections are in themselves stressful events to subjects and may interfere with results. Fourteen-days old pups are as yet pre-weaning, thus administering a chronic corticosterone treatment to experimental mouse mothers through food may elevate corticosterone levels in milk without physically interfering with the pups. However, it is difficult using this paradigm to control

efficient corticosterone concentrations and achieve the same levels as a 3-day MS paradigm would produce.

(2) *HATs inhibitor administration*. HATs are critical enzymes for histone acetylations. As enhanced histone acetylations were observed in the present study, our epigenetic hypothesis should ideally be confirmed by blockage of HATs. However, HAT inhibitors are not like HDAC inhibitors, which are well-developed and widely used in pharmacology studies, there are very few developed HAT inhibitors used in basic research. C646 is documented as a p300/CBP specific inhibitor (one of HATs inhibitors). There is only one in vivo study in neuroscience filed indicating that locally injecting C646 into the PFC improved fear extinction memory (Marek et al., 2011). Moreover, the application of C646 in our model would be blighted with the same problems as discussed above regarding corticosterone administration. However, studies have found that HDACs inhibitors showed anti-depressant effects in behavioural tests (Covington et al., 2009; Iii et al., 2011) with possible involvement in mechanisms of antidepressant-like effect (Yamawaki et al., 2012). If the function of HDACs is simply considered as an opposing function of HATs, then we should expect that HAT inhibitors block all effects we observed in the present study, including elevated histone acetylations and resulting increased Arc and Egr1 gene expression, increased dendritic structures and enhanced emotional and cognitive behaviours.

The present study only targeted to two synaptic plasticity genes of interest, rather than screening for all genes that might be altered in response to early life stress, one cannot therfore exclude consideration of other genes whose expression are associated with histone acetylations, such as genes coding for traditional transmitters' receptors, for example, 5-HT receptors, GABA receptors and dopamine receptors, genes coding for GR, CRF receptors, neurotrophines and other factors which were introduced in Chapter 1.3. In addition, some key mediators may be a target for future studies, CaMKs for example, and its function in the possible mechnisms of regulating HAT activity. A preliminary study in our lab using gene arrays indicates that there is an increased CaMK 2g in the hippocampus of MS pups, which is consistent with the plausible mechanisms discussed above.

Another issue should be considered in the future study whether there are long term stable effects and heritability. The classic definition of epigenetics implies alternations induced by chromatin remodelling without changing DNA sequence, which should maintain and be transmitted from generation to generation without the original stimulus. The present study

only investigated in immediate alternations of histone acetylations after MS. However, we don't know whether these effects maintain or are transmitted to their pups. In fact, whether histone modifications are truly epigenetic is still being argued (For a review, see Kouzarides, 2007).

6. Reference

Abraham A and Gruss M (2010) Stress inoculation facilitates active avoidance learning of the semi-precocial rodent Octodon degus. Behav Brain Res 213:293–303.

Allis C. David, Jenuwein T and Reinberg D (2009) Epigenetics. Cold Spring Harbor, New York. Cold Spring Harbor Laboratory Press.

Alme MN, Wibrand K, Dagestad G and Bramham CR (2007) Chronic fluoxetine treatment induces brain region-specific upregulation of genes associated with BDNF-induced long-term potentiation. Neural Plast 2007:26496.

Aniko K and Baram TZ (2011) Plasticity of the stress response early in life: Mechanisms and significance. Dev Psychobiol 52:661–670.

Anon (2008) Prenatal exposure to maternal depression, neonatal methylation of human glucocorticoid receptor gene (NR3C1) and infant cortisol stress responses. Epigenetics 3:97–106.

Asia B, Tordera R, Lasheras B, Del Río J and Ramírez MJ (2007) Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. Psychoneuroendocrinology 32(3):256-266.

Babygirija R, Yoshimoto S, Gribovskaja-Rupp I, Bülbül M, Ludwig K and Takahashi T (2012) Social interaction attenuates stress responses following chronic stress in maternally separated rats. Brain Res 1469:54-62.

Brake WG, Flores G, Francis D, Meaney MJ, Srivastava LK and Gratton A (2000) Enhanced nucleus accumbens dopamine and plasma corticosterone stress responses in adult rats with neonatal excitotoxic lesion to the medial prefrontal cortex. Neuroscience 96:687-695.

Belsky J, Jonassaint C, Pluess M, Stanton M, Brummett B and Williams R (2009) Vulnerability genes or plasticity genes? Mol Psychiatry 14:746–754.

Bito H, Deisseroth K and Tsien RW (1996) CREB phosphorylation and dephosphorylation: a Ca(2+)- and stimulus duration-dependent switch for hippocampal gene expression. Cell 87:1203–1214.

Boccia ML and Pedersen CA (2001) Brief versus long maternal separations in infancy: contrasting relationships with adult maternal behavior and lactation levels of aggression and anxiety. Psychoneuroendocrinology 26:657-672.

Bock J, Gruss M, Becker S and Braun K (2005a) Experience-induced changes of dendritic spine densities in the prefrontal and sensory cortex: correlation with developmental time windows. Cereb Cortex 15:802–808.

Bock J, Thode C, Hannemann O, Braun K and Darlison MG (2005b) Early socio-emotional experience induces expression of the immediate-early gene Arc/arg3.1 (activity-regulated cytoskeleton-associated protein/activity-regulated gene) in learning-relevant brain regions of the newborn chick. Neuroscience 133:625–633.

Bock J, Murmu MS, Biala Y, Weinstock M and Braun K (2011) Prenatal stress and neonatal handling induce sex-specific changes in dendritic complexity and dendritic spine density in hippocampal subregions of prepubertal rats. Neuroscience 193:34-43.

Bock J, Riedel A and Braun K (2012) Differential changes of metabolic brain activity and interregional functional coupling in prefrontao-limbic pathways during different stress conditions: functional imaging in freely behaving rodent pups. Front Cell Neurosci 6:19 doi:10.3389.

Boudaba C, Szabó K and Tasker JG (1996) Physiological mapping of local inhibitory inputs to the hypothalamic paraventricular nucleus. J Neurosci 16:7151–7160.

Boudaba C, Schrader LA and Tasker JG (1997) Physiological Evidence for Local Excitatory Synaptic Circuits in the Rat Hypothalamus. J Neurophysiol 77:3396–3400.

Boyle MP, Brewer J a, Funatsu M, Wozniak DF, Tsien JZ, Izumi Y and Muglia LJ (2005) Acquired deficit of forebrain glucocorticoid receptor produces depression-like changes in adrenal axis regulation and behaviour. Proc Natl Acad Sci U S A 102:473–478.

Bozon B, Davis S and Laroche S (2002) Regulated transcription of the immediate-early gene Zif268: Mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. Hippocampus 12(5):570-577.

Bozon B, Davis S and Laroche S (2003) A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. Neuron 40:695–701.

Bramham CR, Worley PF, Moore MJ and Guzowski JF (2008) The immediate early gene arc/arg3.1: regulation, mechanisms, and function. J Neurosci 28(46):11760-11767.

Bramham CR, Alme MN, Bittins M, Kuipers SD, Nair RR, Pai B, Panja D, Schubert M, Soule J, Tiron A and Wibrand K (2010) The Arc of synaptic memory. Exp Brain Res 200:125–140.

Cai Q, Zhang B, Huang S, Wang T and Zhou T (2011) The effects of prenatal stress on expression of CaMK-II and L-Ca 21 channel in offspring hippocampus. Acta Biochim Biophys Sin 43:601–606.

Carlezon W a, Duman RSand Nestler EJ (2005) The many faces of CREB. Trends Neurosci 28:436–445.

Calfa G, Chapleau CA, Campbell S, Inoue T, Morse SJ, Lubin FD and Pozzo-Miller L (2012) HDAC activity is required for BDNF to increase quantal neurotransmitter release and dendritic spine density in CA1 pyramidal neurons. Hippocampus 22:1493-1500.

Castellano JF, Fletcher BR, Kelley-Bell B, Kim DH, Gallagher M and Rapp PR (2012) Age-related memory impairment is associated with disrupted multivariate epigenetic coordination in the hippocampus. PloS one 7:e33249.

Charmandari E, Tsigos C and Chrousos G (2005) Endocrinology of the stress response. Annu Rev Physiol 67:259-284.

Chawla S, Vanhoutte P, Arnold FJL, Huang CL-H and Bading H (2003) Neuronal activity-dependent nucleocytoplasmic shuttling of HDAC4 and HDAC5. J Neurochem 85:151–159.

Checkley S (1996) The neuroendocrinology of depression and chronic stress. Br Med Bull 52(3):597-617.

Chen DY, Bambah-Mukku D, Pollonin G and Alberini CM (2012) Glucocorticoid receptors recruit the CaMKIIα-BDNF-CREB pathways to mediate memory consolidation. Nat Neurosci 15(12):1707-14.

Chen J, Evans a N, Liu Y, Honda M, Saavedra JM and Aguilera G (2012) Maternal Deprivation in Rats is Associated with Corticotrophin-Releasing Hormone (CRH) Promoter Hypomethylation and Enhances CRH Transcriptional Responses to Stress in Adulthood. J Neuroendocrinol 24:1055–1064.

Chen Y, Bender R a, Brunson KL, Pomper JK, Grigoriadis DE, Wurst W and Baram TZ (2004) Modulation of dendritic differentiation by corticotropin-releasing factor in the developing hippocampus. Proc Natl Acad Sci U S A 101:15782–15787.

Cole AJ, Saffen DW, Baraban JM and Worley PF (1989) Rapid increase of an immediate early gene messenger RNA in hippocampal neurons by synaptic NMDA receptor activation. Nature 340(6233):474-476.

Covington HE, Maze I, LaPlant QC, Vialou VF, Ohnishi YN, Berton O, Fass DM, Renthal W, Rush AJ, Wu EY, Ghose S, Krishnan V, Russo SJ, Tamminga C, Haggarty SJ and Nestler EJ (2009) Antidepressant actions of histone deacetylase inhibitors. J Neurosci 29:11451–11460.

Cryan JF, Markou A and Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. Trends Pharm Sci 23:238–245.

Cryan JF, Mombereau C and Vassout A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacologcial and genetic studies in mice. Neurosci Biobehav Rev 29(4-5):571-625.

Cullinan WE, Herman JP, Battaglia DF, Akil H and Watson SJ (1995) Pattern and time course of immediate early gene expression in rat brain following acute stress. Neuroscience 64:477–505.

Curzon G (1988) Serotonergic mechanisms of depression. Clin Neuropharmacol 11 Suppl 2:S11-20.

Daniels W, Fairbairn L, Tilburg G van, McEvoy C, Zigmond M, Russell V and Stein D (2009) Maternal separation alters nerve growth factor and corticosterone levels but not the DNA metylation status of the exon 1(7) glucocorticoid receptor promoter region. Metab Brain Dis 24:615–627.

D'Agostino J, Vaeth GF and Henning SJ (1982) Diurnal rhythm of total and free concentrations of serum corticosterone in the rat. Acta Endocrinol (Copenh) 100(1):85-90.

Davis S, Bozon B and Laroche S (2003) How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? Behav Brain Res 142:17–30.

Day JJ and Sweatt JD (2011) Review Epigenetic Mechanisms in Cognition. Neuron 70:813–829.

Deisseroth K, Bito H and Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89–101.

Dick DM (2011) Gene-environment interaction in psychological traits and disorders. Ann Rew Clin Psycho 7:383–409.

Diorio D, Viau V and Meaney MJ (1993) The role of the medail prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci 13:3839-3847.

Dudley KJ, Li X, Kobor MS, Kippin TE and Bredy TW (2011) Epigenetic mechanisms mediating vulnerability and resillence to psychiatric disorders. Neurosci Biobehav Rev 35:1544-1551.

Eiland L and Mcewen BS (2012) Early Life Stress Followed by Subsequent Adult Chronic Stress Potentiates Anxiety And Blunts Hippocampal Structural Remodeling. Hippocampus 22:82–91.

Elizalde N, Pastor PM, Garcia-García AL, Serres F, Venzala E, Huarte J, Ramírez MJ, Del Rio J, Sharp T and Tordera RM (2010) Regulation of markers of synaptic function in mouse models of depression: chronic mild stress and decreased expression of VGLUT1. J Neurochem 114(5):1302-1314.

Evans GW and English K (2002) The environment of poverty: multiple stressor exposure, psychophysiological stress, and socioemotional adjustment. Child Dev 73(4):1238-48.

Fass DM, Reis S a, Ghosh B, Hennig KM, Joseph NF, Zhao W-N, Nieland TJF, Guan J-S, Groves Kuhnle CE, Tang W, Barker DD, Mazitschek R, Schreiber SL, Tsai L-H and Haggarty SJ (2013) Crebinostat: A novel cognitive enhancer that inhibits histone deacetylase activity and modulates chromatin-mediated neuroplasticity. Neuropharmacology 64:81–96.

Faulk C and Dolinoy DC (2011) Timing is everything: The when and how of environmentally induced changes in the epigenome of animals. Epigenetics 6:791–797.

Femenía T, Gómez-Galán M, Lindskog M and Magara S (2012) Dysfunctional hippocampal activity affects emotion and cognition in mood disorders. Brain research:1–13.

Fischer A, Sananbenesi F, Wang X, Dobbin M and Tsai LH (2007) Recovery of learning and memory is associated with chromatin remodelling. Nature 447:178-182.

Fitzgerald PB, Laird AR, Maller Jand Daskalakis ZJ (2008) A Meta-Analytic Study of Changes in Brain Activation in Depression. Hum Brain Mapp 29:683–695.

Fone KCF and Porkess MV (2008) Behavioural and neurochemical effects of post-weaning social isolation in rodents-Relevance to developmental neuropsychiatric disorders. Neurosci Biobehav Rev 32:1087-1102.

Fumagalli F, Pasini M, Frasca A, Drago F, Racagni G and Riva MA (2009) Prenatal stress alters glutamatergic system responsiveness in adult rat prefrontal cortex. J Neurochem 109:1733–1744.

Francis DD, Diorio J, Plotsky PM and Meaney MJ (2002) Environmental enrichment reverses the effects of maternal separation on stress reactivity. J Neurosci 22(18):7840-7843.

Franklin TB, Russig H, Weisse IC, Gräff J, Linder N, Michalon A, Vizi S and Mansuy IM (2010) Epigenetic transmission of the impact of early stress aross generations. Biol Psychiary 68:408-415.

Gao S-F and Bao A-M (2011) Corticotropin-releasing hormone, glutamate, and γ-aminobutyric acid in depression. The Neuroscientist 17:124–144.

Gareth MT and Richard LH (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173-183.

George ED, Bordner KA, Elwafi HM and Simen AA (2010) Maternal separation with ealy weaning: a novel mouse model of early life neglect. BMC Neuroscience 11:123-137.

Geyer MA and Markou A (1995) Animal modles of psychiatric disorders. Psychopharmacology:The Fourth Generation of Progress (Bloom FE and Kupfer DJ, eds) 787-798, Raven Press.

Goldwater DS, Pavlides C, Hunter RG, Bloss EB, Hof PR, McEwen BS and Morrison JH (2009) Structural and functional alternations to rat medial prefrontal cortex following chronic restraint stress and recovery. Neuroscience 164(2):798-808.

Gorman JM and Docherty JP (2012) A Hypothesized Role for Dendritic Remodeling in the Etiology of Mood and Anxiety Disorders. J Neuropsychiatry Clin Neurosci 22:256–264.

Gos T, Bock J, Poeggel G and Braun K (2008) Stress-induced synaptic changes in the rat anterior cingulate cortex are dependent on endocrine developmental time windows. Synapse 62(3):229-232.

Gräff J and Mansuy IM (2008) Epigenetic codes in cognition and behaviour. Behav Brain Res 192:70-87.

Gräff J, Rei D, Guan J-S, Wang W-Y, Seo J, Hennig KM, Nieland TJF, Fass DM, Kao PF, Kahn M, Su SC, Samiei A, Joseph N, Haggarty SJ, Delalle I and Tsai L-H (2012) An epigenetic blockade of cognitive functions in the neurodegenerating brain. Nature 483:222–226.

Gruss M, Braun K, Frey JU and Korz V (2008) Maternal separation during a specific postnatal time window prevents reinforcement of hippocampal long-term petentiation in adolescent rats. Neuroscience 152(1):1-7.

Guan J-S, Haggarty SJ, Giacometti E, Dannenberg J-H, Joseph N, Gao J, Nieland TJF, Zhou Y, Wang X, Mazitschek R, Bradner JE, DePinho R a, Jaenisch R and Tsai L-H (2009) HDAC2 negatively regulates memory formation and synaptic plasticity. Nature 459:55–60.

Guzowski JF, McNaughton BL, Barnes C a and Worley PF (1999) Environment-specific expression of the immediate-early gene Arc in hippocampal neuronal ensembles. Nat Neurosci 2:1120–1124.

Guzowski JF, Setlow B, Wagner EK and McGaugh JL (2001) Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of immediate-early genes Arc, c-fos, and zif268 J Neurosci 21:5089-5099.

Halligan SL, Herbert J, Goodyer I and Murray L (2007) Disturbances in morning cortisol secretion in association with maternal postnatal depression predict subsequent depressive symptomatology in adolescents. Biol Psychiatry 62:40–46.

Harris A and Seckl J (2011) Glucocorticoid, prenatal stress and the programming of disease. Horm Behav 59(3):279-289.

Harris KM, Jensen FE and Tsao B (1992) Three-dimensional structure of dendritic spines and synapses in rat hippocampus (CA1) at postnatal day 15 and adult ages: implications for the maturation of synaptic physiology and long-term potentiation, 12(7):2685-2705.

Heim C and Nemeroff CB. (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. Biol Psychiatry 49(12):1023-39.

Henry C, Kabbaj M, Simon H, Moal ML and Maccari S (1994) Prenatal stress increases the Hypothalamo-pituitary-adrenal axis response in yong and adult rats. J Neuroendocrinol 6:341-345.

Hinwood M, Tynan RJ, Day T a and Walker FR (2011) Repeated social defeat selectively increases δFosB expression and histone H3 acetylation in the infralimbic medial prefrontal cortex. Cereb cortex 21:262–271.

Hobara T, Uchida S, Otsuki K, Matsubara T, Funato H, Matsuo K, Suetsugi M and Watanabe Y (2010) Altered gene expression of histone deacetylases in mood disorder patients. J Psychiatr Res 44:263–270.

Hollis F, Wang H, Dietz D, Gunjan A and Kabbaj M (2010) The effects of repeated social defeat on long-term depressive-like behaviour and short-term histone modifications in the hippocampus in male Sprague-Dawley rats. Psychopharmacology 211:69–77.

Hollis F, Duclot F, Gunjan A and Kabbaj M (2012) Individual differences in the effect of social defeat on anhedonia and histone acetylation in the rat hippocampus. Horm Behav 59:331–337.

Holtmaat A, Wilbrecht L, Knott GW, Welker E and Svoboda K (2006) Experience-dependent and cell-type-specific spine growth in the neocortex. Nature 441:979–983.

Holtmaat AJGD, Trachtenberg JT, Wilbrecht L, Shepherd GM, Zhang X, Knott GW and Svoboda K (2005) Transient and persistent dendritic spines in the neocortex in vivo. Neuron 45:279–291.

Hsieh J and Eisch AJ (2010) Neurobiology of Disease Epigenetics , hippocampal neurogenesis , and neuropsychiatric disorders : Unraveling the genome to understand the mind. Neurobiol Dis 39:73–84.

Huang F, Chotiner JK and Steward O (2007) Actin polymerization and ERK phosphorylation are required for Arc/Arg3.1 mRNA targeting to activated synaptic sites on dendrites. J Neurosci 27:9054–9067.

Huhman KL (2006) Social conflict models: can they inform us about human psychopathology? Horm Behav 50(4):640-646.

Hulshof HJ, Novati A, Sgoifo A, Luiten PG, den Boer JA and Meerlo P (2011) Maternal separation decreases adult hippocampal cell proliferation and impairs cognitive performance but has litter effect on stress sensitivity and anxiety in adult Wistar rats. Behav Brain Res 216(2):552-560.

Huntley GW (2012) Synaptic circuit remodelling by matrix metalloproteinases in health and disease. Nat Rev Neurosci 13(11):743-757.

Huot RL, Thrivikraman KV, Meaney MJ and Plotsky PM (2001) Development of adult ethanol preference and anxiety as a consequence of neonatal maternal separation in Long Evans rats and reversal with antidepressant treatment. Psychopharmacology (Berl) 158(4):366-373.

Iii HEC, Vialou VF, Laplant QC, Ohnishi YN and Nestler EJ (2011) Hippocampal-dependent antidepressant-like activity of histone deacetylase inhibition. Neurosci Lett 493:122–126.

Jacobson L and Sapolsky R (1991) The role of the hippocampus in feedback regulation of the hypothalamic-pituitary-adrenocortical axis. Endocr Rev 12:118–134.

Jafari M, Seese RR, Babayan AH, Gall CM and Lauterborn JC (2012) Glucocorticoid receptors are localized to dendritic spines and influence local actin signaling. Mol Neurobiol 46(2):304-15.

James AB, Conway A-M and Morris BJ (2005) Genomic profiling of the neuronal target genes of the plasticity-related transcription factor -- Zif268. J Neurochem 95:796–810.

Jones MW, Errington ML, Frenche PJ, Fine A, Bliss TVP, Garel S, Charnay P, Bozon B, Laroche S and Davis S (2001) A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memory. Nat Neurosci 4(3):289-296.

Karatsoreos IN and McEwen BS (2011) Psychobiological allostasis: resistance, resilience and vulnerability. Trends Cogn Sci 15:576–584.

Keller S Sarchiapone M, Zarrilli F, Videtic A, Carli V, Sacchetti S, Lembo F, Angiolillo A, Jovanovic N, Pisanti F, Tomaiuolo R, Monticelli A, Balazic J, Roy A, Marusic A, Cocozza S, Fusco A, Bruni CB, Castaldo G and Chiariotto L (2010) Promoter Methylation in the Wernicke Area of Suicide Subjects. Arch Gen Psychiatry 67:258–267.

Kesner RP (2007) Behavioural functions of the CA3 subregion of the hippocampus. Learn Mem 14:771–781.

Keyes KM, Hatzenbuehler ML and Hasin DS (2011) Stressful life experiences, alcohol consumption, and alcohol use disorders: the epidemiologic evidence for four main types of stressors. Psychopharmacology 218:1–17.

Khoury L, Tang Y, Bradley B, Cubells J and Ressler K (2010) Substance Use, Childhood Traumatic Experience and Post-traumatic Stress Disorder in an Urban Civilian Population. Depress Anxiety 27:1077–1086.

Kitanishi T, Ikegaya Y, Matsuki N and Yamada MK (2009) Experience-dependent, rapid structural changes in hippocampal pyramidal cell spines. Cereb Cortex 19:2572–2578.

De Kloet ER, Ratka A, Reul JM, Sutanto W and Van Eekelen J a (1987) Corticosteroid receptor types in brain: regulation and putative function. Ann NY Acad Sci 512:351–361.

Klose RJ and Bird AP (2006) Genomic DNA methylation: the mark and its mediators. Trends Biochem Sci 31:89–97.

Knapska E and Kaczmarek L (2004) A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? Prog Neurobiol 74:183–211.

Ko J, Kim S, Chung HS, Kim K, Han K, Kim H, Jun H, Kaang B-K and Kim E (2006) SALM synaptic cell adhesion-like molecules regulate the differentiation of excitatory synapses. Neuron 50:233–245.

Kolb B, Mychasuik R, Muhammad A, Li Y, Frost DO and Gibb R (2012) Experience and the developing prefrontal cortex. Proc Natl Acad Sci U S A 109 Suppl2:17186-17193.

Komatsuzaki Y, Hatanaka Y, Murakami G, Mukai H, Hojo Y, Saito M, Kimoto T and Kawato S (2012) Corticosterone induces rapid spinogenesis via synaptic glucocorticoid receptors and kinase networks in hippocampus. PloS One 7(4):e34124.

Koolhaas JM, De Boer SF, De Rutter AJ, Meerlo P and Sgoifo A (1997) Social stress in rats and mice. Acta Physiol Scand Suppl. 640:69-72.

Kouzarides T (2007) Chromatin modifications and their function. Cell 128:693–705.

Kozyrskyj AL, Bahreinian S and Azad MB (2011) Early life exposures: impact on asthma and allergic disease. Curr Opin Allergy Clin Immunol 11:400–406.

Kupfer DJ, Frank E and Phillips ML (2011) Major depressive disorder: new clinical, neurobiological, and treatment perspectives. Lancet 379:1045–1055.

Labonte B, Yerko V, Gross J, Mechawar N, Meaney MJ, Szyf M and Turecki G (2012) Differential glucocorticoid receptor exon 1(B), 1(C), and 1(H) expression and methylation in suicide completers with a history of childhood abuse. Biol Psychiatry 72:41–48.

Ledoux JE (2000) Emotion circuits in the brain. Annu Rev Neurosci 23:155–184.

Lambás-Señas L, Mnie-Filali O, Certin V, Faure C, Lemoine L, Zimmer L and Haddjeri N (2009) Functional correlates for 5-HT(1A) receptors in maternally deprived rats displaying anxiety and depression-like behaviors. Prog Neuropsychopharmacol Biol Psychiatry 33(2):262-268.

Lee JH, Kim HJ, Kim JG, Ryu V, Kim BT, Kang DW and Jahng JW (2007) Depressive behaviors and decreased expression of serotonin reuptake transporter in rats that experienced neonatal maternal separation. Neurosci Res 58(1):32-39.

Lee PR, Brady D and Koenig JI (2003) Corticosterone alters N-methyl-D-aspartate receptor subunit mRNA expression before puberty. Brain Res Mol Brain Res 115:55–62.

Lee S, Doulames V, Donnelly M, Levasseaur J and Shea TB (2012) Environmental enrichment can prevent cognitive decline induced by dietary oxidative challenge. J Alzheimers Dis 28(3):497-501.

Leonard BE (2007) Psychopathology of depression. Drugs Today (Barc) 43(10):705-16.

Lesch KP and Waider J (2012) Serotonin in the modulation of neural plasticity and networks: implications for neurodevelopmental disorders. Neuron 76(1):175-191.

Leuner B, Falduto J and Shors TJ (2003) Associative memory formation increases the observation of dendritci spines in the hippocampus. J Neurosci 23:659–665.

Levenson JM, O'Riordan KJ, Brown KD, Trinh M a, Molfese DL and Sweatt JD (2004) Regulation of histone acetylation during memory formation in the hippocampus. J Biol Chem 279:40545–40559.

Levenson J and Sweatt J (2005) Epigenetic mechanisms in memory formation. Nat Rew Neurosci 6(2):108-118.

Levine A, Worrell TR, Zimnisky R and Schmauss C (2012) Early life stress triggers sustained changes in histone deacetylase expression and histone H4 modifications that alter responsiveness to adolescent antidepressant treatment. Neurobiol Dis 45:488–498.

Li L, Carter J, Gao X, Whitehead J and Tourtellotte WG (2005) The Neuroplasticity-Associated Arc Gene Is a Direct Transcriptional Target of Early Growth Response (Egr) Transcription Factors. Mol Cell Biol 25(23):10286–10300.

Liston C, Miller MM, Goldwater DS, Radley JJ, Rocher AB, Hof PR, Morrison JH and McEwen BS (2006) Stress-induced alterations in prefrontal cortical dendritic morphology predict selective impairments in perceptual attentional set-shifting. The Journal of neuroscience : the official journal of the Society for Neuroscience 26:7870–7874.

Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A, Sharma S, Pearson D, Plotsky PM and Meaney MJ (1997) Maternal care, hippocampal gulcocorticoid receptors, and hypothalamic-pituitray-adrenal responses to stress. Science 277:1659-1662.

Liu D, Diorio J, Day JC, Francis DD and Meaney MJ (2000) Maternal care, hippocampal synaptogenesis and cognitive development in rats. Nat Neurosic 3:799-806.

Liu X, Lou M and Wu K (2012) Epigenetic interplay of histone modifications and DNA methylation mediated by HDA6. Plant Signal Behav 7(6):633-5.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the ^{2}-ΔΔCT method. Methods 25(4):402-8.

Lucki I (1997) The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. Behav Pharmocol 8:523-532.

Lupien SJ, McEwen BS, Gunnar MR and Heim C (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nature reviews Neuroscience 10:434–445.

Lyford GL, Yamagata K, Kaufmann WE, Barnes C a, Sanders LK, Copeland NG, Gilbert DJ, Jenkins N a, Lanahan a a and Worley PF (1995) Arc, a growth factor and activity-regulated gene, encodes a novel cytoskeleton-associated protein that is enriched in neuronal dendrites. Neuron 14:433–445.

Maccari S and Morley-Fletcher S (2007) Effects of prenatal restraint stress on the hypothalamus-pituitary-adrenal axis and related behavioural and neurobiological alterations. Psychoneuroendocrinology Suppl 1:S10-5.

MacQueen GM, Ramakrishnan K, Ratnasingan R, Chen B and Young LT (2003) Desipramine treatment reduces the long-term behavioural and neurochemical sequelae of early-life maternal separataion. Int J Neuropsychopharmacol 6(4):391-396.

Makena N, Bugrith K and Russell VA (2012) Maternal separation enhances object location memory and prevents exercise-induced MAPK/ERK signalling in adult Sprague-Dawley rats. Metab Brain Dis 27(3):377-85.

Mangiavacchi S, Masi F, Scheggi S, Leggio B, De Montis MG and Gambarana C (2001) Long-term behavioral and neurochemical effects of chronic stress exposure in rats. J Neurochem 79(6):1113-1121.

Marek R, Coelho CM, Sullivan RKP, Baker-Andresen D, Li X, Ratnu V, Dudley KJ, Meyers D, Mukherjee C, Cole P a, Sah P and Bredy TW (2011) Paradoxical enhancement of fear extinction memory and synaptic plasticity by inhibition of the histone acetyltransferase p300. J Neurosci 31:7486–7491.

Maria A (1996) Chronic Psychosocial Stress Hippocampal CA3 Pyramidal Causes Apical Dendritic Neurons in Subordinate Atrophy of Tree Shrews. J Neurosci 76:3534–3540.

Martisova E, Aisa B, Guereñu G, Ramírez MJ (2012) Effects of early maternal separation on biobehavioral and neuropathological markers of Alzheimer's disease in adult male rats. Curr Alzheimer Res Online

Matrisciano F, Tueting P, Dalal I, Kadriu B, Grayson DR, Davis JM, Nicoletti F and Guidotti A (2012) Epigenetic modifications of GABAergic interneurons are associated with the

schizophrenia-like phenotype induced by prenatal stress in mice. Neuropharmacology:1–11.

McClelland S, Korosi A, Cope J, Ivy A and Baram TZ (2011) Neurobiology of Learning and Memory Emerging roles of epigenetic mechanisms in the enduring effects of early-life stress and experience on learning and memory. Neurobiol Learn Mem 96:79–88.

McEwen BS, De Kloet ER and Rostene W (1986) Adrenal steroid receptors and actions in the nervous system. Physiol Rev 66:1121–1188.

McEwen BS and Gianaros PJ (2010) Central role of the brain in stress and adaptation: links to socioeconomic status, health, and disease. Ann NY Acad Sci 1186:190–222.

McEwen BS, Eiland L, Hunter RG and Miller MM (2012) Stress and anxiety: structural plasticity and epigenetic regulation as a consequence of stress. Neuropharmacology 62:3-12.

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, Turecki G and Meaney MJ (2009a) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 12:342–348.

McGowan PO, Sasaki A, D'Alessio AC, Dymov S, Labonté B, Szyf M, Turecki G and Meaney MJ (2009b) Epigenetic regulation of the glucocorticoid receptor in human brain associates with childhood abuse. Nat Neurosci 12:342–348.

McKinsey T a, Zhang CL and Olson EN (2001) Control of muscle development by dueling HATs and HDACs. Curr Opin Genet Dev 11:497–504.

Meaney MJ, Diorio J, Francis D, Weaver S, Yau J, Chapman K andSeckl JR (2000) Postnatal handling increases the expression of cAMP-inducible transcription factors in the rat hippocampus: the effects of thyroid hormones and serotonin. J Neurosci 20:3926–3935.

Meaney MJ and Szyf M (2005) Environmental programming of stress responses through DNA methylation: life at the interface between a dynamic environment and a fixed genome. Dialogues Clin Neurosci 7(2):103-123.

Meaney MJ and Ferguson-Smith AC (2010) Epigenetic regulation of the neural transcriptome: the meaning of the marks. Nat Neurosci 13:1313-1318.

Mikkelsen JD and Larsen MH (2006) Effects of stress and adrenalectomy on activity-regulated cytoskeleton protein (Arc) gene expression. Neurosci Lett 403:239–243.

Miller EK and Cohen JD (2001) An integrative theory of prefrontal cortex function. Annu Rev Neurosci 24:167–202.

Millstein RA, Ralph RJ, Yang RJ and Holmes A (2006) Effects of repeated maternal separation on prepulse inhibition of startle across inbred mouse strains. Genes Brain Behav 5:346-354.

Millstein RA and Holmes A (2007) Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. Neuroscie Biobehav Rew 31:3–17.

Mizoguchi K, Shoji H, Ikeda R, Tanaka Y and Tabira T (2008) Persistent depressive state after chronic stress in rats is accompanied by HPA axis dysregulation and reduced prefrontal dopaminergic neurotransmission. Pharmacol Biochem Behav 91(1):170-5.

Molteni R, Calabrese F, Mancini M, Racagni G and Riva M a (2008) Basal and stress-induced modulation of activity-regulated cytoskeletal associated protein (Arc) in the rat brain following duloxetine treatment. Psychopharmacology 201:285–292.

Monroy E, Hernández-Torres E and Flores G (2010) Maternal separation disrupts dendritic morphology of neurons in prefrontal cortex, hippocampus, and nucleus accumbens in male rat offspring. J Chem Neuroanat 40:93–101.

Muhammad A and Kolb B (2011) Maternal separation altered behaviour and neuronal spine density without influencing amphetamine sensitization. Behav Brain Res 223:7–16.

Muhammad A, Carroll C and Kolb B (2012) Stress during development alters dendritic morphology in the necleus accumbens and prefrontal cortex. Neuroscience 216:103-109.

Mulligan CJ, Stees J and Hughes DA (2012) Methylation changes at NR3C1 in newborns associate with maternal prenatal stress exposure and newborn birth weight. Epigenetics 7:853–857.

Murgatroyd C, Patchev AV, Wu Y, Micale V, Bockmühl Y, Fischer D, Holsboer F, Wotjak CT, Almeida OFX and Spengler D (2009) Dynamic DNA methylation programs persistent adverse effects of early-life stress. Nat Neurosci 12:1559–1566.

Murmu MS, Salomon S, Biala Y, Weinstock M, Braun K and Bock J (2006) Changes of spine density and dendritic complexity in the prefrontal cortex in offspring of mothers exposed to stress during pregnancy. Eur J Neurosci 24(5):1477-1487.

Nugent NR, Tyrka AR, Carpenter LL and Price LH (2011) Gene-environment interactions: early life stress and risk for depressive and anxiety disorders. Psychopharmacology 214:175–196.

Oda M a and Huttenlocher PR (1974) The effect of corticosteroids on dendritic development in the rat brain. Yale J Biol Med 47:155–165.

Okuno H, Akashi K, Ishii Y, Yagishita-Kyo N, Suzuki K, Nonaka M, Kawashima T, Fujii H, Takemoto-Kimura S, Abe M, Natsume R, Chowdhury S, Sakimura K, Worley PF and Bito H (2012) Inverse synaptic tagging of inactive synapses via dynamic interaction of Arc/Arg3.1 with CaMKIIβ. Cell 149:886–898.

Ons S, Martí O and Armario A (2004) Stress-induced activation of the immediate early gene Arc (activity-regulated cytoskeleton-associated protein) is restricted to telencephalic areas in the rat brain: relationship to c-fos mRNA. Journal of neurochemistry 89:1111–1118.

Owen D and Mattews SG (2007) Repeated maternal glucocorticoid

Pascual R and Zamora-León SP (2007) Effects of neonatal maternal deprivation and postweaning enrionmental complexisty on dendritic morphology of prefrontal pyramidal neurons in the rat. Acta Neurobiol Exp (Wars) 67(4):471-479.

Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Cota P, Wittnam JL, Gogol-Doering A, Opitz L, Salinas-Riester G, Dettenhofer M, Kang H, Farinelli L, Chen W and Fischer A (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science 328:753–756.

Philbert J, Pichat P, Palme R, Belzung C and Griebel G (2012) The CRF receptor antagonist SSR125543 attenuates long-term cognitive deficit induced by acute inescapable stress in mice, independently from the hypothalamic pituitary adrenal axis, Pharmacol Biochem Behav 102(3):415-422.

Plotsky PM and Meaney MJ (1993) Early, postnatal experience alters hypothalamic corticotropin-releasing factor(CRF) mRNA, median eminece CRF content and stress-induced release in adult rats. Mol Brain Res 18:195-200.

Pollak DD, Rey CE and Monje FJ (2010) Rodent models in depression research: classical strategies and new directions. 42(4):252-264.

Porsolt RD, Le Pichon M and Jalfre M (1977) Depression: a new animal model sensitive to andtidepressant treatments. Nature 266(5604):730-732.

Pryce TM, Bettschen D, Nanz-Bahr NI and Feldon J (2003) Comparison the effects of early handing and early deprivation on conditioned stimulus, comtext, and spatial learning and memory in adult rats. Behav Neurosci 117(5):883-893.

Pynoos RS, Ritzmann RF, Steinberg AM, Goenjian A and Prisecaru I (1996) A behavioral animal model of posttraumatic stress disorder featuring repeated exposure to situational reminders. 39(2):129-134.

Radley JJ, Sisti HM, Hao J, Rocher a B, McCall T, Hof PR, McEwen BS and Morrison JH (2004) Chronic behavioural stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. Neuroscience 125:1–6.

Ramírez-Amaya V, Vazdarjanova A, Mikhael D, Rosi S, Worley PF and Barnes C a (2005) Spatial exploration-induced Arc mRNA and protein expression: evidence for selective, network-specific reactivation. J Neurosci 25:1761–1768.

Revest JM, Di Blasi F, Kitchener P, Rougé-Pont F, Desmedt A, Turiault M, Tronche F and Piazza PV. (2005) The MAPK pathway and Egr-1 mediate stress-related behavioral effects of glucocorticoids. Nat Neurosci 8(5):664-72.

Ricobaraza A, Cuadrado-Tejedor M, Marco SP, Pérez-Otaño I. and García-Osta A (2012) Phenylbutyrate rescues dendritic spine loss associated with memory deficits in a mouse model of Alzheimer disease. Hippocampus 22:1040-1050.

Ridder S, Chourbaji S, Hellweg R, Urani A, Zacher C, Schmid W, Zink M, Hörtnagl H, Flor H, Henn F a, Schütz G and Gass P (2005) Mice with genetically altered glucocorticoid receptor expression show altered sensitivity for stress-induced depressive reactions. J Neurosci 25:6243–6250.

Rodríguez JJ, Davies H a, Silva a T, De Souza IEJ, Peddie CJ, Colyer FM, Lancashire CL, Fine A, Errington ML, Bliss TVP and Stewart MG (2005) Long-term potentiation in the rat dentate gyrus is associated with enhanced Arc/Arg3.1 protein expression in spines, dendrites and glia. Eur J Neurosci 21:2384–2396.

Roozendaal B, Hernandez A, Cabrera SM, Hagewoud R, Malvaez M, Stefanko DP, Haettig J and Wood MA (2010) Membrane-associated glucocorticoid activity is necessary for modulation of long-term memory via chromatin modification. J Neurosci 30:5037-5046.

Rosenfeld P, Suchecki D and Levine S (1992) Multifactorial regulation of the hypothalamic-pituitary-adrenal axis during development. Neurosci Biobehav Rev 16(4):553-568.

Roth TL, Zoladz PR, Sweatt JD and Diamond DM (2011) Epegenetic Modification of Hippocampal Bdnf DNA in Adult Rats in an Animal Model of Post-Traumatic Stress Disorder. J Psychiatr Res 45:919–926.

Rowan A (2006) Undoing epigenetics. Nat Rev Neurosci 7:doi:10.1038/nrn1832.

Ryan B, Musazzi L, Mallei A, Tardito D, Gruber SH, El Khoury A, Anwyl R, Racagni G, Mathé AA, Rowan MJ and Popoli M (2009) Remodelling by early-life stress of NMDA receptor-dependent synaptic plasticity in a gene-environment rat model of depression. Int J Neuropsychopharmacol 12(4):553-559.

Sales AJ, Biojone C, Terceti MS, Guimarães FS, Gomes MVM and Joca SRL (2011) Antidepressant-like effect induced by systemic and intra-hippocampal administration of DNA methylation inhibitors. Br J Pharmacol 164:1711–1721.

Sananbenesi F and Fischer A (2009) The epigenetic bottleneck of neurodegenerative and psychiatric diseases. Biol Chem 390:1145–1153.

Sanders BJ and Anticevic A (2007) Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats. Behav Brain Res 183:25–30.

Sapolsky RM (2000) Glucocorticoids and hippocampal atrophy in neuropsychiatric disorders. Arch Gen Psychiatry 57:925–935.

Saruta J, Sato S and Tsukinoki K (2010) The role of neurotrophins related to stress in saliva and salivary glands. Histol Histopathol 25(10):1317-1330.

Schiltz C a, Kelley AE and Landry CF (2007) Acute stress and nicotine cues interact to unveil locomotor arousal and activity-dependent gene expression in the prefrontal cortex. Biol Psychiatry 61:127–135.

Schmidt MV, Wang XD and Meijer OC (2011) Early life stress paradigms in rodents: potential animal models of depression? Psychopharmacology 214:131-140.

Schoneveld OJLM, Gaemers IC and Lamers WH (2004) Mechanisms of glucocorticoid signalling. Biochimica et biophysica acta 1680:114–128.

Schroeder M, Sultany T and Weller A (2012) Prenatal stress effects on emotion regulation differ by genotype and sex in prepubertal rats. Dev Psychobiol doi: 10.10.

Schäble S, Poeggel G, Braun K and Gruss M (2007) Long-term consequences of early experience on adult avoidance learning in female rats: role of the dopaminergic system. Neurobio Learn Mem 87:109–122.

Sexton CE, Mackay CE and Ebmeier KP (2012) A Systematic Review and Meta-Analysis of Magnetic Resonance Imaging Studies in Late-Life Depression. Am J Geriatr Psychiatry:1–13.

Shepherd JD and Bear MF (2011) New views of Arc, a master regulator of synaptic plasticity. Nat Neurosci 14:279–284.

Shires KL and Aggleton JP (2008) Mapping immediate-early gene activity in the rat after place learning in a water-maze: the importance of matched control conditions. Eur J Neurosci 28:982–996.

Simão F, Porto JA and Nunes ML (2012) Effects of enriched environment in spatial learning and memory of immature rats submitted to early undernourish and seizures. Int J Dev Neurosci 30(5):363-7.

Skilbeck KJ, Johnston G a R and Hinton T (2010) Stress and GABA receptors. J Neurochem 112:1115–1130.

Soliman ML, Smith MD, Houdek HM and Rosenberger T a (2012) Acetate supplementation modulates brain histone acetylation and decreases interleukin-1β expression in a rat model of neuroinflammation. J Neuroinflammation 9:51.

Song C, Kanthasamy A, Anantharam V, Sun F and Kanthasamy AG (2010) Environmental Neurotoxic Pesticide Increases Histone Acetylation to Promote Apoptosis in

Dopaminergic Neuronal Cells : Relevance to Epigenetic Mechanisms of Neurodegeneration. Mol Pharmacol 77:621–632.

Sousa RJ, Tannery NH and Lafer EM (1989) In situ hybridization mapping of glucocorticoid receptor messenger ribonucleic acid in rat brain. Mol Endocrinol 3:481–494.

Stanton ME, Gutierrez YR and Levine S (1988) Maternal deprivation potentiates pituitary-adrenal stress responses in infant rats. Behav Neurosci 102(5):692-700.

Sterley T-L, Howells FM and Russell VA (2011) Effects of early life trauma are dependent on genetic predisposition: a rat study. Behav Brain Funct : BBF 7:11.

Stewart MG, Davies HA, Sandi C, Kraev IV, Rogachevsky VV, Peddie CJ, Rpdriguez JJ, Cordero MI, Donohue HS, Gabbott PL and Popov VI (2005) Stress supresses and learning induces plasticity in CA3 of rat hippocampus: a three-dimensional untrastructural study of thorny excrescences and their postsynaptic densities. Neuosci 131(1):43-54.

Strine TW, Dube SR, Edwards VJ, Prehn AW, Rasmussen S, Wagenfeld M, Dhingra S and Croft JB (2012) Associations between adverse childhood experiences, psychological distress, and adult alcohol problems. Am J Health Behav 36(3):408-23.

Sullivan PF, Neale MC, Ph D and Kendler KS (2000) Reviews and Overviews Genetic Epidemiology of Major Depression : Review and Meta-Analysis. Psychiatry: Interpersonal and Biological Processes:1552–1562.

Sun CY, Qi SS, Lou XF, Sun SH, Wang X, Dai KY, Hu SW and Liu NB (2006) Changes of learning, memory and levels of CaMKII, CaM mRNA, CREB mRNA in the hippocampus of chronic multiple-stressed rats. Chin Med J (Engl) 119(2):140-7.

Sun H, Kennedy PJ and Nestler EJ (2012) Epigenetics of the Depressed Brain: Role of Histone Acetylation and Methylation. Neuropsychopharmacology:1–14.

Takahashi LK, Turner JG and Kalin NH (1992) Prenatal stress alters brain catecholaminergic activity and potentiates stress-induced behavior in adult rats. Brain Res 574:131-137.

Takatsuru T, Yoshitomo M, Nemoto T, Eto K and Nabekura J (2009) Maternal separation decreases the stability of mushroom spines in adult mice somatosensory cortex. Brain Res 1294:45-51.

Tata DA (2012) Maternal separation as a model of early stress: effects on aspects of emotional behavior and neuroendocrine function. Hellenic J Psychol 9:84-101.

Thode C, Bock J, Braun K and Darlison MG (2005) The chicken immediate-early gene ZENK is expressed in the medio-rostral neostriatum/hyperstriatum ventrale, a brain region involved in acoustic imprinting, and is up-regulated after exposure to an auditory stimulus. Neuroscience 130:611–617.

Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL and Nestler EJ (2006) Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci 9:519–525.

Tsankova NM, Kumar A and Nestler EJ (2004) Histone modifications at gene promoter regions in rat hippocampus after acute and chronic electroconvulsive seizures. J Neurosci 24:5603–5610.

Tsien JZ, Huerta PT and Tonegawa S (1996) The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell 87:1327-1338.

Turner JD and Muller CP (2005) Structure of the glucocorticoid receptor (NR3C1) gene 5' untranslated region: identification, and tissue distribution of multiple new human exon 1. J Mol Endocrinol 35:283–292.

Tyrka AR, Price LH, Marsit C, Walters OC and Carpenter LL (2012) Childhood adversity and epigenetic modulation of the leukocyte glucocorticoid receptor: preliminary findings in healthy adults. PloS one 7:e30148.

Tzingounis AV and Nicoll R a (2006) Arc/Arg3.1: linking gene expression to synaptic plasticity and memory. Neuron 52:403–407.

Urani A, Chourbaji S and Gass P (2005) Mutant mouse models of depression: candidate genes and current mouse lines. Neurosci Biobehav Rew 29:805–828.

Vaissière T, Sawan C and Herceg Z (2008) Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutat Res 659:40–48.

van Heeringen K (2003) The neurobiology of suicide and suicidality. Can J Psychiatry 48(5):292-300.

Van Oers HJ, de Kloet ER, and Levine S (1998) Early vs, late maternal deprivation differentially alters the endocrine and hypothalamic responses to stress. Brain Res Dev Brain Res 111:245-252.

Vazdarjanova A, McNaughton BL, Barnes C a, Worley PF and Guzowski JF (2002) Experience-dependent coincident expression of the effector immediate-early genes arc and Homer 1a in hippocampal and neocortical neuronal networks. Journal Neurosci 22:10067–10071.

Veenema AH, Blume A, Niederle D, Buwalda B and Neumann ID (2006) Effects of early life stress on adult male aggression and hypothalamic vasopressin and serotonin. Eur J Neurosci 24(6):1711-1720.

Vertes RP (2006) Interactions among the medial prefrontal cortex, hippocampus and midline thalamus in emotional and cognitive processing in the rat. Neuroscience 142:1–20.

Vig R, Gordon JR, Thébaud B, Befus AD and Vliagoftis H (2010) The effect of early-life stress on airway inflammation in adult mice. Neuroimmunomodulation 17(4):229-39.

Walker C-D (2010) Maternal touch and feed as critical regulators of behavioural and stress responses in the offspring. Dev Psychobiol 52:638–650.

Weaver ICG, Cervoni N, Champagne F a, D'Alessio AC, Sharma S, Seckl JR, Dymov S, Szyf M and Meaney MJ (2004) Epigenetic programming by maternal behaviour. Nat Neurosci 7:847–854.

Weaver IGG, Champagne FA, Brown SE, Dymov S, Sharma S, Meaney MJ and Szyf M (2005) Reversal of maternal programming of stress responses in adult offspring through methyl supplementation: altering epigenetic marking later in life. J Neurosci 25(47):11045-11054.

Weaver ICG, D'Alessio AC, Brown SE, Hellstrom IC, Dymov S, Sharma S, Szyf M and Meaney MJ (2007) The transcription factor nerve growth factor-inducible protein a mediates epigenetic programming: altering epigenetic marks by immediate-early genes. J Neurosci 27:1756–1768.

Wei L, David A, Duman RS, Anisman H and Kaffman A (2010) Early life stress increases anxiety-like behaviour in Balbc mice despite a compensatory increase in levels of postnatal maternal care. Horm Behav 57:396–404.

Wei L, Simen A, Mane S and Kaffman A (2012a) Early life stress inhibits expression of a novel innate immune pathway in the developing hippocampus. Neuropsychopharmacology 37:567–580.

Wei Q, Fentress HM, Hoversten MT, Zhang L, Hebda-Bauer EK, Watson SJ, Seasholtz AF and Akil H (2012b) Early-life forebrain glucocorticoid receptor overexpression increases anxiety behaviour and cocaine sensitization. Biol Psychiatry 71:224–231.

Wei Q, Lu X-Y, Liu L, Schafer G, Shieh K-R, Burke S, Robinson TE, Watson SJ, Seasholtz AF and Akil H (2004) Glucocorticoid receptor overexpression in forebrain: a mouse model of increased emotional lability. Proc Natl Acad Sci U S A 101:11851–11856.

Weiss D and Coccaro EF (1997) Neuroendocrine challenge studies of suicidal behaviour. Psychiatr Clin North Am 20(3):563-79.

Weinstock M (1997) Does prenatal stress impair coping and regulation of hypothalamic-pituitary-adrenal axis? Neurosci Biobehav Rev 21(1):1-10.

Welberg LAM, Seckl JR and Holmes MC (2000) Inhibition of 11ß-hydroxysteroid dehydrogenase, the feoto-placental barrier to maternal glucocorticoids, permanently programs amygdala GR mRNA expression and anxiety-like behaviour in the offspring. Eur J Neurosci 12(3):1047-1054.

Wellman CL (2001) Dendritic Reorganization in Pyramidal Neurons in Medial Prefrontal Cortex after Chronic Corticosterone Administration. J Neurobiol 49:245–253.

Wigger A and Neumann ID (1999) Periodic maternal deprivation induces gender dependent alterations in behavioral and neuroendocrine responses to emotional stress in adult rats. Physiol Behav 66:293-302.

Wisden W, Errington ML, Williams S, Dunnett SB, Waters C, Hitchcock D, Evan G, Bliss TV and Hunt SP (1990) Differential expression of immediate early genes in the hippocampus and spinal cord. Neuron 4(4):603-614.

Woolley CS, Gould E and McEwen BS (1990) Exposure to excess glucocorticoids alters dendritic morphology of adult hippocampal pyramidal neurons. Brain Res 531:225–231.

Wright RJ (2012) Stress-related programming of autonomic imbalance: role in allergy and asthma. Chem Immunol Allergy 98:32-47.

Xie T, Wang WP. Jia LJ, Mao ZF, Qu ZZ, Luan SQ and Kan MC (2012) Environmental enrichment restores cognitive deficits induced by prenatal maternal seizure. Brain Res 1470:80-8.

Yamawaki Y, Fuchikama M, Morinobu S, Segawa W, Matsumoto T and Yamawaki S (2012) Antidepressant-like effect of sodium butyrate (HDAC inhibitor) and its molecular mechanism of action in the rat hippocampus. World J Biol Psychiatry 13(6):458-67.

Yao ZH, Zhang JJ and Xie XF (2012) Enriched environment prevents cognitive impairment and Tau hyperphosphorylation after chronic cerebral hypoperfusion. Curr Neurovasc Res 9(3):176-84.

Yehuda R, Southwick SM, Ostroff RB, Mason JW and Giller E Jr (1988) Neuroendocrine aspects of suicidal behaviour. Neurol Clin 6(1):83-102.

Yehuda R and Seckl J (2011) Minireview: Stress-related psychiatric disorders with low cortisol levels: a metabolic hypothesis. Endocrinology 152:4496–4503.

York JM, Blevins NA, Baynard T and Freund GG (2012) Mouse testing methods in psychoneuroimmunology: an overview of how to measure sickness, depressive/anxietal, cognitive, and physical activity behaviors. Methods Mol Biol 934:243-276.

Yuan LW and Gambee JE (2001) Histone acetylation by p300 is involved in CREB-mediated transcription on chromatin. Biochim Biophys Acta 1541:161–169.

Yuste R and Bonhoeffer T (2001) Morphological Changes in Dendritic Spines Associated with Long-Term Synaptic Plasticity. Annual Review of Neuroscience 24:1070–1089.

Zimmerberg B, Rosenthal AJ and Stark AC (2003) Neonatal social isolation alters both maternal and pup behaviors in rats. Dev Psychobiol 42:52-63.

Zimmerberg B and Sageser KA (2011) Comparison of two rodent models of maternal separation on juvenile social behavior. Front Psychiatry 2:39.

Appendix

Animal Care

Bedding	Lignolcel S 8-15	Ssniff spezialitäten GmbH, Soest, NW, Germany
Feed	R/M-H, 10mm	Ssniff spezialitäten GmbH, Soest, NW, Germany

Consumptive Materials

Chemicals

$CaCl_2$	E.Merck, Darmstadt
Deoxychlolic acid	SIGMA-ALDRICH CHEMIE GmbH
EDTA	Carl Roth GmbH
Glycine	Carl Roth GmbH
KCl	Carl Roth GmbH
KH_2PO4	SIGMA-ALDRICH CHEMIE GmbH
LiCl	SIGMA-ALDRICH CHEMIE GmbH
Methanol	Carl Roth GmbH
NaCl	Carl Roth GmbH
NaH_2PO_4	Carl Roth GmbH
$NaHCO_3$	Riedel-de Haen
NaN_3	Carl Roth GmbH
NP40	Calbiochem®
Phenylmethysulfonyl fluoride	SIGMA-ALDRICH CHEMIE GmbH
Roti Load1 Dye	Carl Roth GmbH
SDS	SERVA Electrophoresis GmbH
Sodium acetate	Carl Roth GmbH

Tris	Carl Roth GmbH
Tris-HCl	Carl Roth GmbH
Triton X-100	Ferak Berlin
Tween 20	Carl Roth GmbH

Others

4-15% gradient SDS-PAGE gel	Bio-Rad
Anti-acetyl-Histone H3	Millipore
Anti-acetyl-Histone H4	Millipore
Anti-alpha nucleolin	Abcam
BCA protein assay kit	Novagen®, Merck
DNA Molecular Weight Marker VIII (19-1114 bp)	Roche
Spectra^{TM} Muticolor Low Range Protein ladder	Thermo Scientific
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
MicroAmp Fast 8-Tube Strip, 0.1ml	Applied Biosystems
MicroAmp Fast Optical 96-well Reaction Plate	Applied Biosystems
MicroAmp Optical Adhesive Film	Applied Biosystems
MicroAmp8-Cap Strip	Applied Biosystems
Micrococcal Nuclease	USB Corporation
MinElute Reaction Cleanup Kit	QIAGEN GmbH
nProtein A Sepharase 4 fast flow	GE Healthcare
Phase Lock Gel Light 15ml	5prime GmbH
Phosphatase inhibitor coctail	Roche

Protease inhibitor coctail	Roche
Protein ladder	Fermentas
PVDM membrane	Millipore
RNase Inhibitor	Applied Biosystems
RNeasy Plus Mini Kit	QIAGEN GmbH
Seqenase Version 2.0 DNA Polymerase	USB Corporation
Taq DNA Polymerase	QIAGEN GmbH
TaqMan Gene Expression Assays (Arc)	Applied Biosystems
TaqMan Gene Expression Assays (Egr1)	Applied Biosystems
TaqMan Gene Expression Assays (HPRT1)	Applied Biosystems
TaqMan universal PCR Master Mix	Applied Biosystems
2 x PCR Master Mix	Qiagen

Instruments

Mortar and pestle		VWR international GmbH, Darm-stadt, HE, Germany
Vortexer	Vortex genie-2	Scientific Industries Inc., Bethesda, MD, USA
Plate shaker	VXR	IKA® GmbH & Co.KG, Staufen im Breisgau, BW, Germany
Incubator Shaker	thermomixer comfort, 1.5ml	Eppendorf AG, Hamburg, Germany
	Innova^{TM} 4200	New Brunswick Scientific, EDISON, NJ, USA
Incubator	SA1B22040	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Rotator	SB3	Stuart® Barlowworld Scientific Ltd, UK

Centrifuge	5415R	Eppendorf AG, Hamburg, Germany
	5804R	Eppendorf AG, Hamburg, Germany
NanoPhotometer	NanoPhotometer™	IMPLEN GmbH
Microplate reader	Multiscan FC	Thermo Fisher Scientific Inc., Tow Rivers, WI, USA
	Infinite M200	Tecan Deutschland GmbH
DNA imaging reader	Ingenius syngene Bio Imaging	Syngene (VWR Supplier)
Immunoblotting apparatus	Mini-PROTEAN	Bio-Rad Laboratories (UK) Ltd
Immunoblotting power	peqpower E0303	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Imaging reader	FujiFilm, LAS-1000	Fuji Photo Film Co., Ltd
Thermal cycle (conventional PCR)	Veritis 96-well Fast	Applied Biosystems GmbH, Darm-stadt, HE, Germany
Thermal cycler (real-time PCR)	StepOnePlus	Applied Biosystems GmbH, Darm-stadt, HE, Germany
Intellicage	IntelliCage V1.4	NewBehaviour AG, Zurich, Switzer-land
Microscope	Olympus BX51	Olympus, Germany
Camera	Microfire	Optronics®

Software

Data management and statistics	Microsoft Excel 2008
ELISA	ScanIt Software V 2.5
Intellicage	IntelliCage Plus Software
Scientific graphing	GraphPad Prism 5
Dendritic structure anaylsis	NEUROLUCIDA®, MicroBright-Field, Williston, VT, USA

Buffer Recipes

N-ChIP

MNase reaction buffer	10mM Tris-HCl (pH 8.8) 1mM $CaCl_2$
10x FSB	50mM EDTA 200mM Tris 500mM NaCl Adjust pH to 7.5
Low salt washing buffer	0.1% SDS 1% Triton X-100 2mM EDTA 20mM Tris (pH 8) 150mM NaCl
High salt washing buffer	0.1% SDS 1% Triton X-100 2mM EDTA 20mM Tris (pH 8) 500mM NaCl
Lithium chloride buffer	1% NP40 1% Deoxycholic acid 1mM EDTA (pH 8) 10mM Tris (pH 8) 0.25M LiCl
TE buffer	10mM Tris (pH 8) 1mM EDTA (pH 8)
Elution buffer	0.1M $NaHCO_3$ 1% SDS
10x Proteinase K digestion buffer	1M Tris 50mM EDTA 2% SDS 2M NaCl Adjust pH to 8.0

Nuclei protein extraction

10x PBS (1L)	80g NaCl 2g KCl 14.4g Na_2HPO_4 2.4g KH_2PO_4 Adjust pH to 7.4
TEB	1x PBS 0.5% Triton X-100 2mM phenylmethysulfonyl fluoride 0.02% NaN_3

Immunoblotting

10x TBS (1L)	24g Tris base 88g NaCl Adjust PH to 7.6
TBST (1L)	100ml 10x TBS 10ml 10% tween 20 890 ddH_2O
Running buffer (2L)	6.06g Tris base (25mM) 28.53g glycine (190mM) 2g SDS (0.1%) Adjust pH to 8.3
Transfer buffer (2L)	6.06g Tris base (25mM) 28.53g glycine (190mM) 400ml Methanol (20%) Adjust pH to 8.3

Curriculum Vitae

Personal Data	Lan Xie Leipziger str.44, House 91 39120, Magdeburg Gender: Female Birthday: 02.11.1980 Birthplace: Hunan, China Nationality: Chinese
Promotion	*01.09.2008 -30.06.2013* Department of Zoology and Developmental Biology, Institute of Biology, OVG University, Magdeburg **Dissertation**: Early life stress-induced histone acetylations regulate activation of synaptic plasticity in the hippocampus and the medial prefrontal cortex. Supervisor: PD. Dr. Jörg Bock
Publication	**Xie L**, Korkmaz K, Braun AK, Bock J. (2013) Early stress-induced histone acetylations correlate with activation of the synaptic plasticity genes Arc and Egr1 in the mouse hippocampus. *J. Neurochem.* DOI:10.1111/jnc. 12210 (online) Grealish S, **Xie L**, Kelly M, Dowd E. (2008) Delayed-onset nigrostriatal degeneration with rapid-onset motor impairment following unilateral axonal or terminal injection of 6-hydroxydopamine in the rat. *Brain Res. Bull.* 77(5):312-9.
Education	*Sep. 2006-Oct.2007, Master in Neuropharmacology* Department of Pharmacology, National University of Ireland, Galway **Dissertation**: Development of the behavioural and neuropathological consequences of complete and partial dopaminergic lesions in rats. Supervisor: Dr.Eilis Dowd *Sep.1999-Jun.2003, Bachelor in Pharmacy* Department of Pharmacy, Hunan University of Traditional Chinese Medicine, P.R. China **Dissertation**: Active ingredient contents screening and quantification of compound Danshen injections by using HPLC. Supervisor: Prof. Liubao Peng
Profession	*Jul.2003-Aug.2006, Assistant lecturer* Department of Pharmacology, Hunan Environment-Biological Polytechnic, P.R.China

Acknowledgements

First of all, I am grateful to Prof. Anna Katharina Braun for taking me on this project and her thoughtful comments throughout.

I owe a debt of gratitude to my supervisor, PD Dr. Jörg Bock who directed me from project design to discussions around any problems I encountered. His guidance and patience supported me in overcoming the difficulties I met while conducting experiments. With his instruction, I managed to publish my data and achieve the present dissertation.

I would like to express my deep appreciation to Prof. Kemal Kormaz for technique support in establishing the ChIP-qPCR method employed. I must also attest my thanks to Prof. Oliver Stock and the members of his department. Thank you all for your fantastic help during the period of working on molecular experiments.

I take this opportunity to acknowledge all colleagues whom helped me a lot in the laboratory. Especially Susann, Ute and Daniela who assisted me in experiments; Katrin, Sylvia and Daniela who helped me to cope when dealing with German who don't speak English, or Chinese!

Many thanks to Galway Idol, Dr. Joy Simpson who helped me to polish English language in the present dissertation.

Particularly, I am appreciative for my parents' support in every way. Without their understanding, I could not leave them to go 7000+ km away and have the wonderful and unforgettable life trip I have had in Europe.

Finally, I would like to thank the dear friends I have in Magdeburg. Thanks for the precious time of being with them, I will cherish it always. They are the sweet and caring angel Iris Müller, wikipedia-man Wang Han, gossipy girls Fu Ting and Kiran Sandhu, and thoughtful doctor Xiong Yanyan.

Declaration

I declare that the work presented here is, to the best of my knowledge and belief, original and the result of my own investigation. Where other sources of information have been used, they have been acknowledged. This thesis has not been submitted for a degree in any other university.

The work was done under the guidance of P.D. Dr. Jörg Bock, in institute of biology, Otto-von-Geurik University, Magdeburg.

Lan Xie
18.07.2013, Magdeburg