Postnatal maternal separation affects hippocampal long-term potentiation in adult stressed rats: Molecular and hormonal mechanisms

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Abstract

Early-life stress has been reported to induce a series of emotional and cognitive disorders in adulthood. The present study focused on the effects of maternal separation (MS) from postnatal day (PND) 14 to 16 for everyday 6 hours on hippocampal plasticity with *in vivo* hippocampal long-term potentiation (LTP) as a cellular model of learning and memory formation. Then, the results showed that an acute exposure to elevated-platform-stress (EPF-stress) in 11-week-old male rats resulted in a reinforcement of the dentate gyrus (DG) LTP in control animals (MS-), while this emotional reinforcement of LTP was seriously repressed in maternally separated (MS+) rats. Since steroid hormones and their receptors play critical roles in the effects of early-life stress, we measured hippocampal concentrations of 17β-estradiol, corticosterone and testosterone, as well as gene expression levels of their receptors after LTP-induction/EPF-stress in MS+ and MS- rats. The mRNA levels of mineralocorticoid receptor (MR) and estrogen receptor β (ERβ) were upregulated 1 h after EP-stress in MS- rats, but not in MS+ rats. The application of MR and ERβ antagonists impaired the LTP-reinforcement in control rats, verifying their crucial roles in emotion induced LTP-reinforcement.

According to the long-lasting effects of MS on gene expression, the involvement of epigenetic mechanisms was considered. The application of the DNA methyltransferase (DNMT) inhibitor 5-aza-2′-deoxytidine (5-aza) partly restored the LTP-reinforcement in MS+ rats, accompanied by a retrieval of ERβ- but not MR-mRNA upregulation. In contrast, the application of the histone deacetylase (HDAC) inhibitior trichostatin A (TSA) did not affect LTP-reinforcement. DNA methylation assays then identified that MS+ and MS- rats showed different methylation patterns across CpG sites in the ERβ gene promoter sequence 1 h after EPF-stress. These results indicate a key role of ERβ in early-life-stress-mediated emotionality and emotion-induced late-LTP in adult male rats via DNA methylation mechanisms.

Behavioral tests on the elevated plus maze (EPM) showed a decreased anxiety-like behavior in MS+ rats in the second trial, accompanied by a lower level of serum corticosterone level as compared to MS- rats 15 min after the second trial. These results indicate the desensitization to stressful events during adulthood in MS+ rats, in which the regulation and function of ERβ play a crucial role at the cellular level.

Zusamenfassung

Frühe Stresserfahrungen sind in der Lage, emotionale sowie kognitive Informations-verarbeitungsprozesse bei adulten Individuen zu beeinflussen Die *in vivo* hippokampale Langzeit-Potenzierung (LTP), diente in dieser Studie als ein zelluläres Modell für Lernen und Gedächtnisbildung um die Einflüsse maternaler Separation (MS) vom postnatalen Tag 14 bis 16 für täglich 6 Stunden auf die hippokampale Plastizität zu untersuchen. LTP im Gyrus Dentatus (DG) lässt sich in mindestens zwei Phasen gliedern: eine proteinsynthese-unabhängige frühe Phase und eine proteinsyntheseabhängige späte Phase. Die frühe Phase kann durch akuten Stress in einem bestimmten Zeitraum nach der LTP-Induzierung in eine späte Phase transferiert (verstärkt) werden. Eine solche Verstärkung ließ sich in der vorliegenden Arbeit durch akuten Stress ausgelöst durch den Aufenthalt auf einer erhöhten-Plattform (EPF-Stress) in 11 Wochen alten Kontrolratten (MS-) induzieren, während diese emotionale LTP-Verstärkung in den adulten früh gestressten Ratten (MS+) nicht zu beobachten war. Einflüsse des Steroidhormons Corticosteron und seiner Rezeptoren in der Regulation emotionaler Informationsverarbeitung sind bereits bekannt, die Rolle von Geschlechtshormonen und deren Rezeptoren sind dagegen noch wenig untersucht. Daher wurden die hippokampalen Konzentrationen der Steroidhormone Corticosteron, Testosteron und 17β-estradiol und die Expression ihrer Rezeptoren erfasst. Die mRNA-Konzentrationen des Mineralocorticoid- (MR) und des Östrogen-Rezeptoren β (ERβ) waren 1 Stunde nach LTP-Induzierung/EPF-Stress in MS- Ratten gegenüber MS+ Ratten signifikant erhöht. Die funktionelle Rolle von MR und ERβ in der Verstärkung der LTP wurde durch die Applikation der spezifischen Antagonisten überprüft und bestätigt. Die Inaktivierung von MR führte zu einer unmittelbaren Depotenzierung, die von ERβ zu einer Verminderung der späten Phase der LTP.

Die langfristigen Wirkungen der MS auf die hippokampale Genexpression machte die Rolle epigenetischer Mechanismen wahrscheinlich. Die Applikation des Inhibitors (5-aza-2´-deoxytidine) gegen DNA-Methyltransferase (DNMT) konnte teilweise die von EPF-Stress induzierte LTP-Verstärkung und die Expression von ERβ- aber nicht MR-Genen in MS+ Ratten wieder herstellen, während die Applikation des Inhibitors Trichostatin A gegen die Histonedeacetylase (HDAC) keinerlei Effekte hatte DNA-Methylierungsanalysen ergaben keine Unterschiede zwischen MS- und MS+ Ratten ohne EPF-Stress. Eine Stunde nach EPF-stress zeigten sich jedoch Unterschiede im Muster der methylierten CpG-Stellen der ERβ-Genpromotorsequenz zwischen MS+ und MS- Ratten. MR und ERβ regulieren demnach in

konzertierter Aktion die LTP-Verstärkung und die emotionale Informationsverarbeitung nach akutem Stress. ERβ wirkt dabei besonders auf die späte LTP und spielt wahrscheinlich eine Modulierende Rolle bei der Konsolidierung emotionaler Gedächtnisspuren.

Die Sensitivierung und Etablierung von stressinduzierten Gedächtnisinhalten durch frühkindliche Erfahrungen wird dabei durch epigenetische Mechanismen (DNA-Methylierung) reguliert. Das wird durch Untersuchungen im Erhöhten-Kreuzlabyrinth in adulten Ratten bestätigt. Hier zeigten MS+ Ratten eine geringere Anzeichen von Ängstlichkeit und geringere Corticosteron-konzentrationen im Blut als MS- Ratten.

Glossary

AMPA	2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid	**fEPSP**	Field excitatory postsynaptic potential
AR	Androgen receptor	**GR**	Glucocorticoid receptor
BDNF	brain-derived neurotrophic factor	**HAT**	Histone acetyltranferase
BLA	Basolateral amygdala	**HDAC**	Histone deacetylase
BNST	bed nuclei of stria terminalis	**HFS**	High-frequency stimulation
CA	Cornu ammonis	**HPA**	Hypothalamo-pituitary-adrenal
CaMKII	Ca^{2+}/calmodulin-dependent protein kinases II	**HPG**	Hypothalamo-pituitary-gonadal
CHIP	Chromatin precipitation	**5-HT**	5-Hydroxytryptamine
CREB	cAMP response element-binding	**i.c.v.**	Intracerebroventricular
CRF	corticotropin-releasing factor	**i.p.**	Intraperitoneal
DAT	Dopamine transporter	**LTP**	Long-term potentiation
DG	Dentate gyrus	**MAPK**	Mitogen-activated protein kinase
DMSO	Dimethylsulfoxid	**MBD**	methyl-CpG-binding domain protein
DNMT	DNA methyltransferase	**MeCP2**	methyl CpG binding protein 2
ELISA	enzyme-linked immunosorbent assay	**mEPSC**	miniature excitatory postsynaptic current
EPF	Elevated platform	**mGluR**	Metabotropic glutamate receptor
EPM	Elevated plus maze	**MR**	Mineralocorticoid receptor
ER	Estrogen receptor	**MS**	Maternal separation

NMDA N-methyl-D-aspartic acid

PFC Prefrontal cortex

PKA Protein kinase A

PKC Protein kinase C

PKMζ Protein kinase M zeta

PP1 protein phosphatase 1

PRP Plasticity-related protein

PSA Population spike amplitude

PSD-95 Postsynaptic density-95

SEM Standard error of the mean

SNc substantia nigra pars compacta

TSS Transcriptional start site

Catalog

1. Introduction

1.1 Early-life stress and maternal separation paradigms

1.1.1 Maternal separation paradigms

Epidemiological studies have shown that childhood trauma, such as abuse and neglect, increase the risk for brain disorders, especially developing depression in adulthood (Edwards et al., 2003; Heim et al., 2008). According to clinical studies, early-life stress in humans is associated with the persistent sensitization of hypothalamo-pituitary-adrenal (HPA) axis and autonomic nervous system to mild stress in adulthood (Heim et al., 2000). Because of the limitation of clinical studies on humans, a number of animal models have been established for the purpose of better understanding the long-lasting effects of early-life stressful experiences and the underlying mechanisms. Among them, the maternal separation paradigms (MS), i.e. single or repeated separation of the newborn pups from their dam for a certain time period, are well-established animal models to analyze the etiology of behavioral and brain dysfunctions systematically (Sullivan and Brake, 2003; de Kloet et al., 2005). For humans, parental loss during childhood has been reported to be one of the early-life stressful experiences that can increase the risk of major depression (Kendler et al., 2002) and psychosis (Morgan et al., 2007). Similarly, in rodents and non-human primate models, the disruption of usual mother-infant interactions can induce a series of neuronal, physiological, emotional and behavioral anomalies in adulthood (Hoff et al., 1994; Ellenbroek et al., 1998; Lyons et al., 1998; Rosenblum et al., 2001; Lehmann and Feldon, 2000; Pryce et al., 2005; Joëls and Baram, 2009).

Nevertheless, different separation protocols may lead to different outcomes. Previous studies have revealed the crucial role of time-window and duration of MS paradigm in the psycho-physiological effects (Lehmann et al., 1999; Gos et al., 2008; Gruss et al., 2008). In rodents, MS is performed usually in the first two weeks of the postnatal life. Two experimental protocols are mainly applied: an acute protocol, including a prolonged single episode (24 h) and a chronic protocol with periodic brief periods (3-6 h per days for several days) of MS (Marco et al., 2009). For the present study, the latter protocol was applied. Since the comparison of the different outcomes between different MSprotocols is beyond the scope of this dissertation's topics, this introduction will especially focus on the effects of repeated chronic MS paradigms in rodents.

1.1.2 Effects of maternal separation

Physiological effects The most direct and common effect of various MS paradigms is both short- and long-term upregulation of HPA axis activity, including hypersecretion of plasma adrenocorticotropin and corticosterone in response to stress, increased corticotropin-releasing factor (CRF) mRNA in the paraventricular nucleus (PVN) as well as decreased hippocampal glucocorticoid receptor (GR) expression level (Plotsky and Meaney, 1993; Meaney et al., 1996; Lehmann et al., 1999; Aisa et al., 2007). In humans, hyperactivity of HPA axis, reflected by increased central corticotrophin-releasing factor (CRF) activity, is found to be one of the most remarkable neuroendocrine features of depression (Heim et al., 2002). Besides adrenal steroids and their receptors, MS can also affect the expression of other stress hormones, such as vasopressin (Murgatroyd et al., 2009). In animal models, long-term alteration of HPA axis activity may result in a cascade of neurobiological and behavioral changes (Pryce and Feldon, 2003; Champagne and Curley, 2009).

Neurobiological effects MS has been found to have adverse effects on hippocampal neurons, including the reduction of mossy fiber density and impaired neurogenesis (Huot et al., 2002; Mirescu et al., 2004). Recent data showed that MS from postnatal day (PND) 1 to 10 for everyday 4 h leads to the reduction of dentritic length and spine density in prefrontal cortex (PFC) and CA1 region in the ventral hippocampus (Monroy et al., 2010). Remarkably, decreased hippocampal volume appears also in persons with childhood trauma with or without posttraumatic stress disorder (PTSD) (Bremner et al., 1997; Stein et al., 1997). In contrast to the MS within the first two weeks of postnatal life, MS from postnatal day (PND) 14 to 16 for everyday 1 h can increase the length of dendrites and the density of dendritic spines in prefrontal and sensory cortex (Bock et al., 2005). These changes are supposed to be the result of the overexposure to stress hormones, caused by the MS-induced hyperactivity of HPA axis. A support of this view is that repeated central CRF injection during development results in a decreased hippocampal volume, which can also be induced by MS (Brunson et al., 2001).

At the molecular level, MS affects not only the expression of stress-related proteins like GR and CRH (Plotsky and Meaney, 1993; Meaney et al., 1996; Lehmann et al., 1999), but also other molecules in the nervous system. MS from PND 2 – 14 for everyday 3 h leads to increased brain-derived neurotrophic factor (BDNF) concentration in the hippocampus, but decreased concentration in the striatum (Roceri et al., 2004). Exposure to a MS from PND 5 – 21 for 6 h per day induces an elevation of c-Fos (a transcription factor mainly expressed in recently activated neurons) mRNA level in the cingulate cortex, amygdala and hippocampus

(Troakes and Ingram, 2009). More and more evidences associate the effects of maternal behavior on gene expression with the alteration of epigenetic status of the relevant genes, which may be resulted from the overexposure to glucocorticoids in a sensitive time-window early in life (Weaver et al., 2005; Murgatroyd et al., 2009). This topic will be introduced in more detail in the part on epigenetic mechanisms for synaptic plasticity.

Behavioral and cognitive effects Because of molecular, physiological and morphological alterations within the hippocampus, animals experienced MS often show dysfunctions in spatial and contextual learning as well as memory formation, including impaired spatial learning ability in T-maze, Morris water maze and novel object recognition test (Garner et al., 2007; Aisa et al., 2007). Brief MS impairs the extinction recall of fear conditioning (Wilber et al., 2009), suggesting a deficit in memory formation. This effect is accompanied by increased GR in the infralimbic region of the medial PFC (Wilber et al., 2009). The MS-induced learning and memory deficits are often associated with depression-like behavior, such as increased immobility in forced swimming test as well as anxiety-like behavior in elevated plus maze and startle inducing auditory stimuli (Aisa et al., 2007; Kalinichev et al., 2002). The treament with GR antagonist can reverse the depression-like behavior induced by MS (Aisa et al., 2007), suggesting the interaction among stress, HPA axis, the hippocampus and the amygdala as the major regulation for the development of psychotic disorders (Phillips et al., 2006).

1.2 Hippocampal long-term potentiation

1.2.1 Memory and hippocampus

Memory is a fundamental property of the brain. It can be divided into four processes: encoding, storage, consolidation and recording (Morris et al., 2003). According to thesynaptic plasticity and memory hypothesis, ‘encoding and storage of memory depend on the activity-dependent synaptic plasticity induced at appropriate synapses during memory formation mediated by the brain area in which the plasticity is observed’ (Morris et al., 2003; Andersen et al., 2007). Although the variety of memory systems in the brain makes it difficult to locate a neural correlate to memory, studies in both patients and animals have indicated the key role of the hippocampus in the rapid encoding of declarative memory (Squire, 1992). Animal studies have proved the dependence of spatial-learning tasks on a functional hippocampus and N-methyl-D-aspartic acid (NMDA) receptor (Morris, 1981, 1984; Martin and Clark, 2007). Storage of spatial information as well as a representation of the environment and navigational

functions can be attributed to the hippocampus (Clark et al., 2005) by the cognitive map theory (O'Keefe and Nadel, 1978).

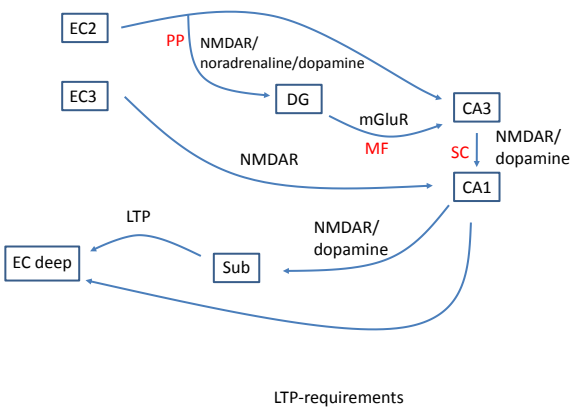

Figure 1. The pathways of the hippocampal formation. The entorhinal cortex gives off the perforant path, projecting to the DG region in the hippocampus. The mossy fibers project from granule cells in the DG to the CA3. The CA3 pyramidal neurons give off the schaffer collateral, projecting to the CA1 region. CA1 sends output to hippocampal formation and deep layers of the entorhinal cortex. EC: entorhinal cortex; MF: mossy fibers; PP: perforant path; SC: schaffer collateral; Sub: subiculum.

The hippocampus is a major component in the brains of human and other vertebrates. The hippocampus is anatomically divided into the dentate gyrus (DG) and cornu ammonis (CA). The DG (it is actually a part of the hippocampal formation, but the most research articles describe its function as a structure of the hippocampus) is composed of the fascia dentata and the hilus, while CA is differentiated into fields CA1, CA2, CA3 and CA4. The main pathways within the hippocampus are known as the "trisynaptic loop". The DG contains the tightly packed granule neurons (mostly interneurons and glia in the hilus, granule cells are only in the small DG layer), while CA contains with the densely packed pyramidal neurons arranged in a even smaller band. The DG receives the excitatory inputs mainly from layers II and IV of the entorhinal cortex. The axons of the perforant path mainly project to layer II and III of the enthorinal cortex. The perforant path provides the main sensory input to the DG. Granule neurons of the DG project their axons called 'mossy fibers' to pyramidal neurons of CA3, which send their axons (Schaffer collaterals) to CA1. The CA1 region sends output to hippocampal formation and deep layers of the entorhinal cortex. In addition, there is a projection from the lateral perforant path to the CA1 and separately from layer III of the enthorinal cortex with the temporal ammonic pathway (fig. 1). Besides entorhinal cortex, the hippocampus also receives the inputs from the amygdala (specifically the anterior amygdaloid area, the basolateral nucleus, and the periamygdaloid cortex) as well as the lateral preoptic and lateral hypothalamic areas and other brain structures (Andersen et al., 2007). The most

widely studied form of hippocampal synaptic plasticity is the long-term potentiation (LTP). The hippocampus is the favorable site for LTP studies because of its densely packed and sharply defined layers of neurons (Cooke and Bliss, 2006).

1.2.2 Hippocampal long-term potentiation as a cellular model for memory formation

Long-term potentiation refers to a long-lasting enhancement in signal transmission between two neurons through stimulating them synchronously (Cooke and Bliss, 2006) and is one of the most important phenomena of synaptic plasticity. LTP was first observed in the hippocampal DG in anesthetized rabbits in 1966, by stimulating the perforant path with a high-frequency train of stimuli (in the following called tetanus) through implanted electrodes (Lomo, 1966). Since memory is thought to be encoded by the modification of synaptic strength (Bliss and Collingridge, 1993), LTP is widely considered as a cellular model for the basic mechanisms for learning and memory formation (Frey and Morris, 1997, 1998).

The best-studied form of LTP, which can be induced *in vivo* and was used in the present study, is the late-associative NMDA-receptor dependent LTP (Reymann and Frey, 2007). NMDA receptor is a dual ligand- and voltage-dependent glutamatergic receptor and serves as a molecular correlate to the coincidence-criterion proposed by Hebb (Hebb, 1949). This dissertation will mainly focus on this form of LTP induced *in vivo* in the DG. As a model for memory formation, the induction and maintenance of associative NMDA-receptor dependent LTP (just called ‘LTP’ in the following) requires the activation and synthesis of plasticity-related proteins (Krug et al., 1984; Frey et al., 1988; Otani and Abraham, 1989; Otani et al., 1989), while this kind of LTP consists of phases (Reymann et al., 1988a; Matthies et al., 1990). These properties are just in line with the hypothesis of the neuronal mechanisms underlying memory formation in the mammalian brain (Matthies, 1974, 1989): ‘memory is divided into three assumed phases, a short-term, an intermediate and a long-term phase, which correspond to their respective different cellular properties at the synaptic, synaptosomal, and nuclear level required for the regulation of memory formation’.

1.2.3 molecular processes of LTP

The *in vivo* induction of LTP in the DG is accomplished by stimulating the medial perforant path fibers, which take their rise from layer II neurons in the entorhinal cortex and terminate on granule cells in the DG. Congruent to memories, LTP also can be divided into a protein-synthesis-independent early-phase lasting 4 – 6 h and a protein-synthesis-dependent late phase lasting more than 8 h or even days. Anisomycin, a reverse translation inhibitor, blocks LTP beyond 4 – 6 h after tetanus (Krug et al., 1984; Frey et al., 1988; Reymann et al., 1988a).

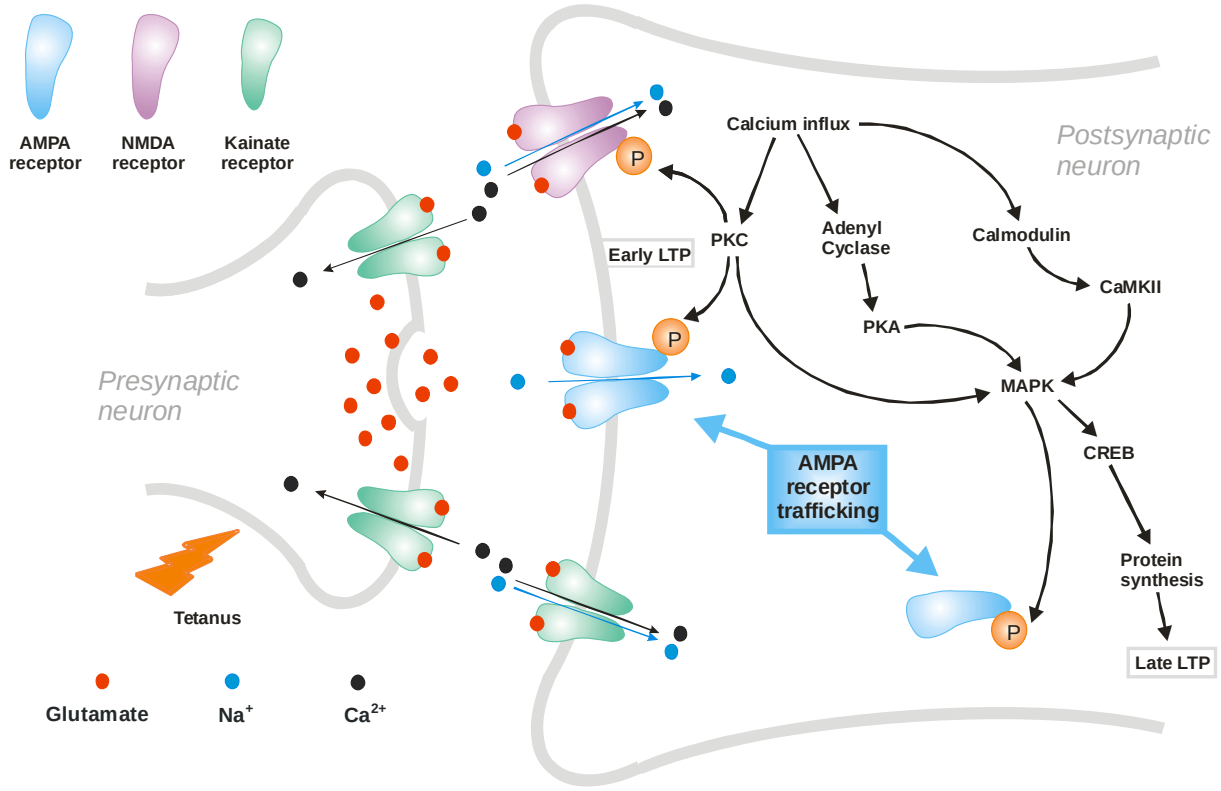

Figure 2. Molecular processes of early and late-LTP. Tetanic stimulation elicits a surge of Ca^{2+} influx into postsynaptic neuron through activated NMDA and AMPA receptors. This Ca^{2+} influx activates then PKC, Adenyl Cyclase and Calmodulin. The activated kinases phosphorylate glutamate receptors, which elevates the conductance of postsynaptic membrane and induces early-LTP. If the tetanic stimulation is strong enough or there are heterosynaptic inputs, MAPK and its downstream transcription factors, such as CREB, will be activated and induce protein synthesis as well as gene expression. The newly synthesized plasticity-related proteins will preserve the hyperconductence of postsynaptic membrane and thus produce late-LTP.

Early-LTP It was already known that the initial phase of LTP is induced by a temporary coactivation of NMDA receptor and 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) receptors during tetanic stimulation and a concurrent influx of Ca^{2+} into the postsynaptic cell via NMDA-receptor-operated channels (Herron et al., 1986). The maintenance of this phase of LTP does not require the synthesis of new proteins, but the modification of already existing proteins (fig. 2). The tetanus-induced surge of Ca^{2+} in postsynaptic cells and the activation of metabotropic glutamate receptor (mGluR) (Ben-Ari et al., 1992; Fitzjohn et al., 1996) activate protein kinase C (PKC) and Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). These two kinases are proved to be necessary for early-LTP (Lovinger et al., 1987; Malinow et al., 1989). The activated kinases can phosphorylate the glutamate receptors (NMDA-, AMPA- and mGlu receptors). This

modification increases their sensitivity and reinforces the synaptic strength transiently (Reymann et al., 1988b; Davies et al., 1989; Soderling and Derkach, 2000).

Late-LTP In contrast to early-LTP, the maintenance of the LTP after 6 h depends on newly synthesized plasticity-related proteins in the postsynaptic cell (fig. 2). The formation of the second messenger cAMP and the activation of cAMP-dependent protein kinase A (PKA) are found to play an essential role for the protein synthesis required for late-LTP. The application of PKA inhibitors is ineffective on early-LTP, but blocks late-LTP (Frey et al., 1993; Matthies and Reymann, 1993), whereas the transient application of membrane-permeable cAMP analog or other PKA activators can initiate a delayed LTP (Frey et al., 1993a; Pockett et al., 1993; Selbach et al., 1997). As the phosphorylation targets of PKA, PKC and CaMKII, mitogen-activated protein kinase (MAPK) and its downstream transcription factor cAMP response element-binding (CREB) play important role in late-LTP induction *in vitro* (Ahmed and Frey, 2005), but there is still no evidence indicating their roles *in vivo*.

Moreover, the cAMP cascade can also be activated by dopaminergic inputs and the consequent activation of dopamine receptors. The hippocampus is innervated by dopamine fibers (Baulac et al., 1986) and dopamine level increases during conventional LTP induction in the CA1 (Frey et al., 1990). The activation of dopamine receptors with the transient application of dopamine or similar agonists can initiate a delayed potentiation with a similar time course of late-CA1-LTP (Gribkoff and Ashe, 1984; Frey and Morris, 1998; Sajikumar and Frey, 2004). These results revealed the key role of heterosynaptic inputs in late-LTP formation.

1.2.4 Reinforcement of early-LTP

Late-LTP can be directly induced by a strong tetanus. However, early-LTP can also be transformed into late-LTP by delayed activation of an independent synaptic input that contributes to late-LTP through inducing the synthesis of plasticity-related proteins (fig. 3) (Reymann and Frey, 2007). This input can be activated by an electrical stimulation at other brain structures or a behavioral challenge on tested animals.

Structural reinforcement If the induction of an early-LTP is coupled with an electrical activation of basolateral amygdala (BLA), early-LTP in the DG can then be reinforced into late-LTP (Frey et al., 2001). This reinforcement effect depends on β-adrenergic and muscarinergic (Frey et al., 2001), but not dopamine receptors, in the DG rather than CA1 region (Manahan-Vaughan and Kulla, 2003; Korz and Frey, 2007). Besides BLA, the

stimulation at the noradrenergic locus coeruleus and the serotonergic raphe nucleus can also affect LTP in the DG (Ezrokhi et al., 1999). Furthermore, the nucleus supramammillaris and the medial septum were implicated to play a role in plastic processes within the DG (Reymann and Frey, 2007).

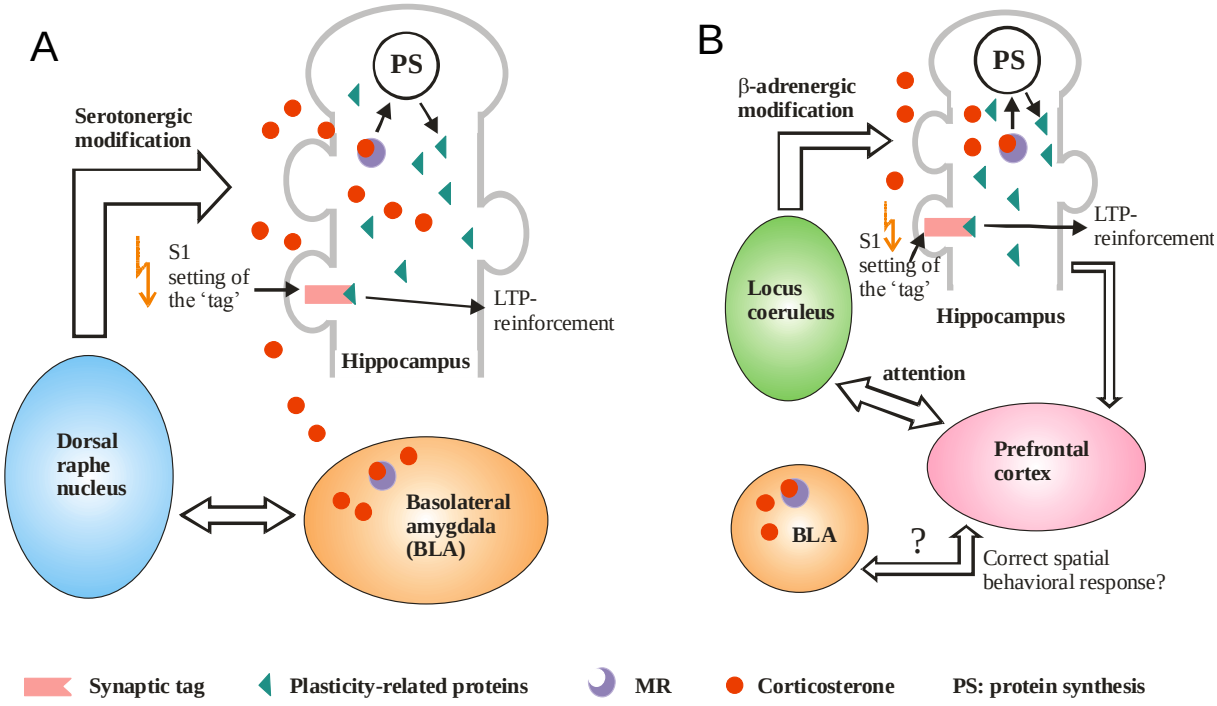

Figure 3. Synaptic tagging hypothesis for behavioral reinforcement of early-LTP *in vivo* (Korz and Frey, 2005a; modified). (A) Emotional reinforcement. The induction of early-LTP sets 'tags' in the stimulated synaptic terminals. An acute stress increases hippocampal corticosterone level and activates MR. The activated MR can promote synthesis of plasticity-related proteins, which connect with synaptic tags and maintain LTP. Protein synthesis also depends on heterosynaptic serotonergic inputs induced by activated basolateral amygdale via the Dorsal raphe. (B) Cognitive reinforcement. Early-LTP can also be transferred into late-LTP by mastering a spatial learning task. In this case, synthesis of plasticity-related proteins depends on not only MR activity but also β-adrenergic activation through the interaction between locus coeruleus and PFC, which may be activated by correct spatial behavioral response.

Behavioral reinforcement An acute stress, like swim stress in a water maze within 15 min to 1 h after tetanization, is able to reinforce early-PSA-LTP into late-LTP in the DG (Korz and Frey, 2003). The corticosterone binding mineralocorticoid receptor (MR) was found to play a crucial role in this reinforcing effect on LTP (Korz and Frey, 2003, 2005b). Recent studies revealed that post-tetanic-stress-induced DG-LTP reinforcement depends on serotonergic but not dopaminergic heterosynaptic receptor activation (Ahmed et al., 2006)

(fig. 3 A), which probably mediates the BLA-dependent modification of LTP under stress (Frey et al., 2001).

Besides emotional challenges, cognitive components are also able to induce the transformation from early- to late-LTP. Early-LTP can be reinforced into late-LTP by associating it with holeboard training in a critical time window within the learning curve (Korz and Frey, 2004; Uzakov et al., 2005). It was found that the consolidation of a spatial reference memory after training corresponds to the induction of protein-synthesis-dependent LTP (fig. 3 B), which suggests that the consolidation of reference memory and LTP maintenance, namely the long-lasting enhancement of synaptic strength, may share at least a part of the newly synthesized proteins (Korz and Frey, 2004).

Synaptic tagging theory The above-mentioned reinforcement effects on LTP can be explained by the "synaptic tagging hypothesis" proposed by Morris and Frey (Frey and Morris, 1997) to explain the mechanisms underlying the input-specificity property of LTP. According to this hypothesis, the newly potentiated synapses are tagged by some local proteins. The newly synthesized plasticity-related proteins (PRPs) can only be detected in the tagged synapses through the interaction between the PRPs and the synaptic tags. However, the tag is transiently activated with an expected half-life of about 30 min in slices (probably even longer in the intact animal), explaining why the protein synthesis induced by the heterosynaptic inputs is effective on LTP-reinforcement only in a certain time window around tetanization (Frey and Morris, 1997, 1998). With respect to early-LTP reinforcement, electrical stimulation at BLA, stress and learning tasks induce respective heterosynaptic inputs to hippocampal neurons. These inputs result in a cascade of activation of receptors and kinases. The upregulation of CaMKII- and MAPK2-mRNA was found 3 h after one-trial aversive learning (Müller Igaz et al., 2004). This cascade finally activates the synthesis of PRPs, which are then captured by the tags in the newly potentiated synapses, resulting in the prolonged enhancement of synaptic transmission efficacy (fig. 3).

1.3 The involvement of steroid hormones and their receptors in hippocampal plasticity

1.3.1 Steroid hormone and their receptors

Steroid hormones are steroids that act as hormones. They contain 5 groups according to the receptors they bind: glucocorticoids, mineralocorticoids, androgens, estrogens and progestogens. All of them are synthesized from cholesterol. According to their different functions and sites of synthesis, they can be assigned to sex hormones (gonadal hormones),

including androgens and estrogens, as well as stress hormones (adrenocortical hormones), and stress hormones (adrenal hormones), including glucocorticoids and mineralocorticoids. Progestogens are precursors of other steroid hormones. In humans and rodents, the most important derivatives of androgens and estrogens are testosterone and estradiol (Goodman, 2009). Moreover, a subgroup of steroid hormone neuroactive steroids has been found to interact with neurotransmitter-gated ion channels such as $GABA_A$ (Majewska et al., 1986; Herd et al., 2007), NMDA (Wu et al., 1991) and sigma receptors (Maurice et al., 1997) and modulate their activity.

The main receptors for steroid hormones are GR, MR (both of them bind with corticosterone; the latter with a 10 fold higher affinity), androgen receptor (AR) binding with testosterone and estrogen receptor (ER), which has two subtypes: ERα and ERβ. Since steroid hormones are permeable through cell membrane, these receptors are mainly located in cytosol and nucleus acting as transcription factors (Goodman, 2009). But in recent years, more and more transmembrane and extracellular receptors were identified (Norman et al, 2004). Steroid hormone receptors widely distribute in various tissues in mammals, including nervous system (Goodman, 2009). Hippocampal neurons express all of the main types of steroid hormone receptors, which have been found to play important roles in hippocampal synaptic plasticity.

1.3.2 Hippocampus as a target of stress

The hippocampus was the first higher brain center that was recognized as a target of stress hormones and expresses receptors for both types of adrenal steroids (McEwen et al., 1968). The activation of MR is proved to be necessary both for LTP maintenance in CA1 in hippocampal slices (Pavlides et al., 1996) and stress-induced LTP reinforcement in the DG *in vivo* (Korz and Frey, 2003), whereas GR activation shows opposing roles to MR in LTP induction and reinforcement (Pavlides et al., 1995; Korz and Frey, 2003; Avital et al., 2006). Their different roles are thought to be attributed to their different affinities to corticosteroids. MR binds corticosterone with a higher affinity, approximately 10-fold higher than colocolized GR (Veldhuis et al., 1982; Krozowski and Funder, 1983). Thus, hippocampal MRs respond more quickly but shortly to corticosterone, whereas the slower activation of GRs mediates feedback actions aimed to restore disturbances in homeostasis (de Kloet and Reul, 1987). The concerted activation of MR and GR in succession from low to high concentration of corticosterone can modify neuronal function profoundly (Dallman et al., 1992; Joëls and de Kloet, 1992).

Besides LTP, adrenocortical hormones and their receptors also affect other forms of synaptic plasticity. In the hippocampus, chronic restraint stress leads to retraction and simplification of dendrites in the CA3 region (McEwen, 1999; Sousa et al., 2000). Moreover, certain types of acute stress and chronic stressors can regulate neurogenesis or apoptosis in the DG (Gould et al., 1997). The circuitry of the DG-CA3 system is thought to play a role in the memory of sequences of events, although the storage of long-term memory occurs in other brain regions (Lisman and Otmakhova, 2001). This structural remodeling is shown to be mediated by adrenal steroids (Magarinos et al., 1999; Sousa et al., 2000) and extracellular excitatory amino acids such as glutamate (McEwen, 1999). As a consequence of hippocampal structural remodeling mentioned above, chronic restraint stress also induces corresponding impairment of spatial memory formation (Luine et al., 1994), whereas an acute reduction of corticosterone can conteract this impairment (Wright et al., 2006).

1.3.3 Hippocampus as a target of sex hormones

Not only adrenal steroids but also gonadal steroids are involved in hippocampal plasticity and memory formation. In female rats, the synaptogenesis in CA1 during the estrous cycle depends on estrogens and NMDA receptor (McEwen et al., 1995; Woolley, 1999). Estradiol treatment increases NMDA receptor R1 subunit expression both in cell body and dendrites (Gazzaley et al., 1996). This effect is likely mediated by cholinergic activity through inhibitory GABAergic interneurons (Daniel and Duhanich, 2001; Rudick and Woolley, 2001; Rudick et al., 2003). Besides nuclear receptors, estrogens also have membrane-associated receptors coupled with second messenger systems (Razandi et al., 1999; McEwen and Milner, 2007). Previous studies have reported an estrogenic rapid effect that enhances phosphylation of CREB in cell nuclei in hippocampal pyramidal neurons (Lee et al., 2000). This effect is likely mediated by nonnuclear ERs (McEwen, 2010).

Androgens and their receptors also show modulatory effects on hippocampal synaptic plasticity. Studies in both rodents and non-human primates demonstrated that gonadoectomy in males results in significant decrease in the density of dendritic spine synapses in CA1 (Leranth et al., 2003, 2004a,b). In addition, progesterone receptors are also expressed in the hippocampus in axons, dendrites, synaptic terminals as well as glial cell processes and play a role in hippocampal plasticity (Parsons et al., 1982; Waters et al., 2008). The application of progesterone is found to downregulate spine density in CA1 neurons (Woolley, 1999).

1.3.4 Interaction between different types of steroid hormones in hippocampal plasticity

The interaction between stress and sex hormones as well as their receptors is mainly reflected by the sex differences in stress effects. For instance, chronic restraint stress for 21 days has been reported to enhance or have no effect on female rats' spatial learning ability, but negatively influences males in spatial learning tasks (Luine et al., 1994; Bowman et al., 2001, 2003). Similar chronic stress has difference effects on cell proliferation in the DG between males and females. Chronic restraint stress induces retraction of dendrites in the CA3 region of the hippocampus in males, but not in females, unless the females are ovariectomized (Galea et al., 1997). On the other hand, exposure to acute stress suppresses learning ability in female rats, whereas the same stressful event can enhance learning ability in males (Wood and Shors, 1998). This sex difference disappears when the hippocampus is damaged (Bangasser and Shors, 2007), suggesting the hippocampus as a critical target of the interaction between stress and sex hormones. Sex-dependent effects of neonatal MS have been reported in behavior and neuroendocrine in adult rats (McIntosh et al., 1999; Wigger and Neumann, 1999; Slotten et al., 2006).

These sex differences in stress-responsiveness and processing is mainly due to the regulatory effects of Hypothalamo-pituitary-gonadal (HPG) axis on HPA axis (McCormick and Mathews, 2007). During proestrus, the phase of estrous cycle with high level of estradiol, females have higher corticosterone level than during the other phases of estrous cycle (Raps et al., 1970; Bohler et al., 1990; Walker et al., 2001). Neonatally castrated male rats with testosterone replacement in adulthood have a higher level of corticosterone release in response to stress than adult-castrated males with the same testosterone replacement (McCormick et al., 1998). This result suggests that neonatal testosterone influences the ability of sex hormones to regulate HPA function in adulthood (McCormick and Mathews, 2007). Furthermore, i.c.v. infusion of testosterone can impair cognitive reinforcement of LTP, which is also MR-dependent (Schulz and Korz, 2010a). Besides the interaction between stress and sex hormones, different sex hormones were also found to interact with each other. Castration is known to increase ERα expression in male rats' brain (Handa et al., 1996). This result indicates the involvement of testosterone in the regulation of ER expression in the nervous system.

1.4 Epigenetical mechanisms for synaptic plasticity

1.4.1 Molecular basis of epigenetic mechanisms

Epigenetics refers to the study on the changes of gene expression induced by the mechanisms other than changes in DNA sequence. The main mechanisms of non-genetic regulation of gene expression are chromatin remodeling and DNA methylation (Razin, 1998) (fig. 4). The basic building block of chromatin is the nucleosome, which is made up of an octamer of histones. Chromatin remodeling is achieved by post-transcriptional modification of the N-terminal tail of histones, including acetylation, methylation, ubiquitylation, sumoylation as well as phosphorylation (McGowan and Szyf, 2010).

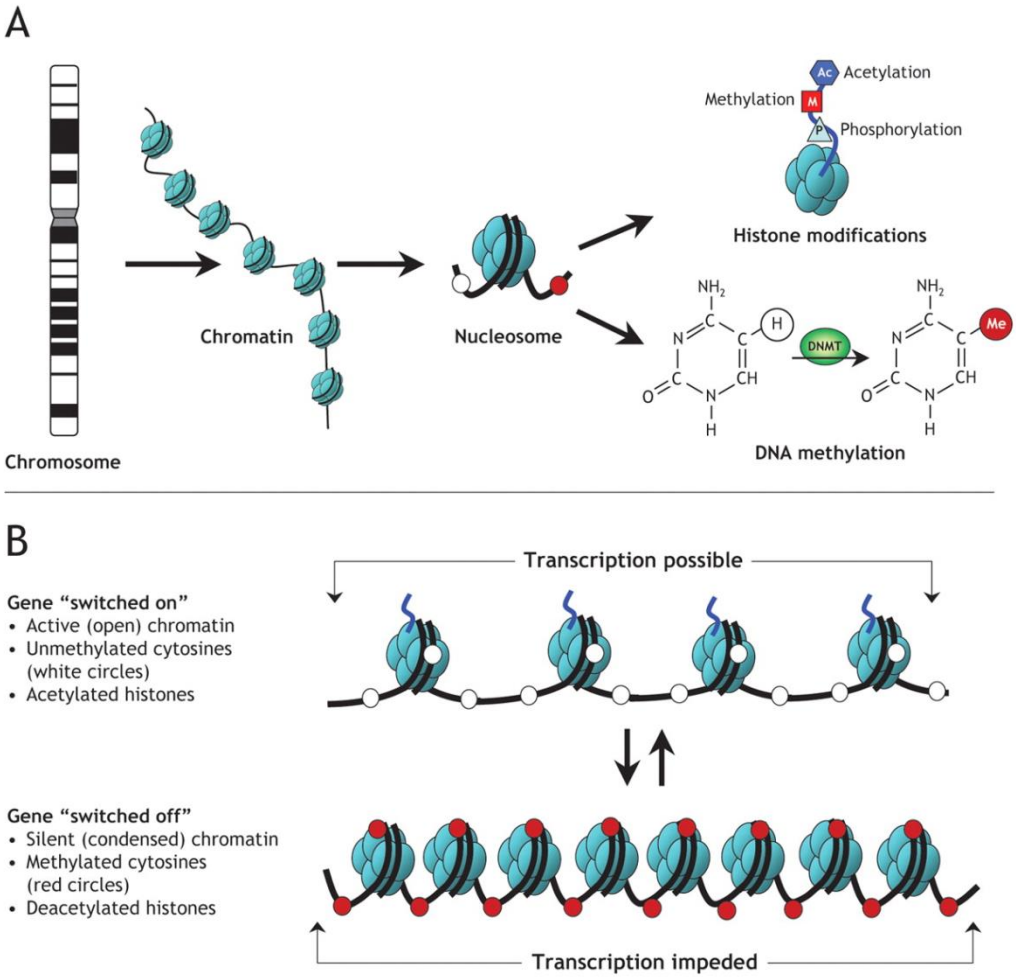

Figure 4. Covalent modification of DNA and histones are the main forms of epigenetic mechanisms (cited from http://sfbtrr58.uni-muenster.de/typo3temp/pics). (A) DNA methylation is achieved by adding methyl groups on cytosine with the catalysis of DNMT, while histones can be modified by acetylation, methylation, and phosphorylation. (B) Histone acetylation induces a loose configuration of chromatin enhancing gene expression, whereas gene transcription can be "switched off" by histone deacetylation and DNA methylation.

Acetylation is the most highly studied form of histone modifications. The acetylation of histones H3 and H4 is catalyzed by histone acetyltransferase (HAT). Acetylated tails lower the charge of histones and loose the association between histones and DNA backbone. This loose configuration is believed to enhance the accessibility of a gene to the transcription machinery. Conversely, histone deacetylase (HDAC) catalyses histone deacetylation and results in a tight binding between histones and DNA backbone, which limits the accessibility of genes to transcription factors (Kuo and Allis, 1998).

The second way of epigenetic regulation of gene expression is the addition of methyl groups to DNA, mostly at CpG sites, which convertscytosine to 5-methylcytosine. This modification is catalyzed by DNA methyltransferase (DNMT). Methylation in gene promoters and enhancers can prevent these sequences from being recognized and bound by transcription factors, then silence gene transcription (McGowan and Szyf, 2010). DNA methylation can also repress gene expression indirectly with attraction of methylated-DNA-binding proteins such as MeCP2, which recruits histone-modifying enzymes to tighten chromatin configuration and silence gene expression (Nan et al., 1997).

1.4.2 Emotional and cognitive induction of epigenetic alterations

Recent studies have indicated that changes of epigenetic status can be induced by stress and learning. Exposure of rodents to forced swimming significantly increases the number of the cells with acetylated H3 in the DG (Bilang-Bleuel et al., 2005; Chandramohan et al., 2007). Histone H3 acetylation is enhanced in the hippocampus following contextual fear conditioning (Levenson et al., 2004, 2006; Lubin and Sweatt, 2007). Not only histone acetylation, fear conditioning can also upregulate DNMT gene expression in adult rats' hippocampus and this upregulation was associated with rapid methylation of gene *protein phosphatase 1* (Miller and Sweatt, 2007). These findings suggest an essential role of epigenetic events in stress responsiveness and the regulation of memory formation.

Maternal behavior is able to induce long-lasting changes of epigenetic modifications in the offspring. The offspring of low licking and grooming dams shows lower level of hippocampal GR gene expression and corresponding higher methylation level within the promoter region of the GR gene (Weaver et al., 2004). Remarkably, lower level of maternal licking and grooming is also associated with high level of ERα methylation in female offspring (Champagne et al., 2006). It has been mentioned above that MS from PND 1 to 12 for everyday 3 h increases the expression level of arginine vasopressin gene in the hypothalamus paraventricular nucleus. This effect was proved to depend on DNA hypomethylation of an

important regulation region of arginine vasopressin gene (Murgatroyd et al., 2009). According to these findings, epigenetic modifications have been widely accepted as one of most important mechanisms underlying the MS-induced phenotypes in adulthood.

1.5 Aim and design of the present study

Although previous studies have revealed a great number of MS-induced anormalies in hippocampal plasticity, few of results have indicated the effects of MS on hippocampal LTP *in vivo* and the molecular mechanisms underlying these effects. Therefore, the present study designed the following experiments to reveal the effects and mechanisms.

Experiment I: Effects of MS on hippocampal LTP

The MS paradigm used in the present study is based on the results of a study published in 2005 by Bock et al. This study revealed that MS on rat pups from PND 14 to 16 for everyday 1 h increased the density of dendritic spines and the length of dendrites in pyramidal neurons of anterior cingulate cortex and somatosensory cortex in 21-week-old pups. According to this finding, such a MS paradigm may also affect synaptic plasticity in the hippocampus. Therefore, the present study wanted to reveal how a MS in the same time window induces a long-lasting effect on hippocampal LTP. The time-courses of LTP were compared between maternally separated rats and controls, in order to reveal the effects of MS on the basic electrophysiological properties in the hippocampus. Since MS is able to induce a significant and long-lasting alteration of HPA-axis activity (Champagne and Curley, 2009), the effect of MS on the stress-induced LTP-reinforcement was intensively investigated.

Experiment II: The role of steroid hormones and their receptors in the MS-induced changes of hippocampal LTP

With respect to the mechanisms underlying the effects of MS, the present study focused on steroid hormones and their receptors. Previous studies have been proved the critical role of corticosteroid receptors in emotional reinforcement of hippocampal LTP (Korz and Frey, 2003), while HPA axis can be severely affected by MS (Plotsky and Meaney, 1993; Meaney et al., 1996; Lehmann et al., 1999). These findings suggest stress hormones and their receptors as the potential bridge across early stress and the modifications of LTP. Moreover, sex hormones were also found to be able to regulate synaptic plasticity (McEwen, 2010) and the activity of HPA axis (McComick and Mathews, 2007).

According to these results, we hypothesized that the changes of emotional LTP-reinforcement may be due to MS-induced alteration of steroid hormone level and gene expression of their

receptors in the hippocampus. In order to verify this hypothesis, the hippocampal level of steroid hormones and mRNA level of their receptors were measured and compared between different groups of rats. Then, the antagonists against the receptors whose expression was affected by MS were applied respectively in certain time windows around LTP-induction, so as to reveal the function of steroid hormone receptors in LTP induction and maintenance.

Experiment III: The role of epigenetic mechanisms in the MS-induced changes of hippocampal LTP and gene expression

Since maternal behavior and MS are proved to have effects on epigenetic mechanisms (Weaver et al., 2004; Murgatroyd et al., 2009) and a lot of evidences have indicated the importance of epigenetic mechanisms in synaptic plasticity (Champagne and Curley, 2009), the present study aimed to reveal the epigenetic mechanisms underlying the effects of MS. DNA methylation and histone acetylation are the most highly studied forms of epigenetic modifications. So at first, the inhibitors against DNMT and HDAC were applied respectively. If one of the inhibition treatments was able to counteract the effects of MS on LTP, the effect of the inhibitor on the gene expression of steroid hormone receptors were then investigated. The genes whose expression was altered by the inhibition against DNMT or HDAC were then used for DNA-methylation or histone-acetylation assays, in order to confirm the involvement of epigenetic mechanisms in the effects of MS and reveal the MS-induced epigenetic changes of these genes.

Experiment IV: Effects of MS on behavior and brain morphology

Early-life stress has been widely thought to be a risk factor for depression in adulthood. However, a similar MS paradigm was found to have positive effects on neuronal plasticity (Bock et al., 2005). Therefore, we wanted to reveal how a MS in this time window impacts on depression-like behavior in rats. After behavioral tests, the brain tissue was also collected to make brain slices for the observation of morphological differences between maternally separated rats and controls.

2. Materials

2.1 Rat strain

Male Wistar rats strain Hanover from the breeding colony, at the Institute of Biology, Otto von Guericke University of Magdeburg.

2.2 Bedding material and feed

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

2.3 Instruments

Instruments for electrophysiological surgery

Stereotaxic Frame	Rat configuration	TSE Systems, Midland, MI, USA
Oscilloscope	HM507	Hameg Instruments GmbH, Mainhausen, HE, Germany
Isolated Pulse Stimulator	Model 2100	A-M Systems, Carlsborg, WA, USA
Differential AC Amplifier	INH	Science Products, Hochheim, HE, Germany
Dental Drill	WD56-EM	W&H Dentalwerk Bürmoos, Bürmoos, Austria

Instruments for electrophysiological recording

Recording Box (with swivel and cable)	40×40×40 cm	Leibniz Institute of Neurobiology, Magdeburg, ST, Germany

Isolated Pulse Stimulator	Model 2100	A-M Systems, Carlsborg, WA, USA
Differential AC Amplifier	INH	Science Products, Hochheim, HE, Germany
Analog/Digital Interface	CED 1401+	Cambridge electronic design, Cambridge, UK

Instruments for behavioral tests

Elevated Platform	120 cm high 20×20 cm	Leibniz Institute of Neurobiology, Magdeburg, ST, Germany
Elevated Plus Maze	100 cm high	Institute of Biology, University of Magdeburg, ST, Germany

Instruments for i.c.v. infusion

Balance	Ultramicro Cubis®	Sartorius AG, Göttingen, NI, Germany
Cannula	Ø 700 µm	Leibniz Institute of Neurobiology, Magdeburg, ST, Germany
Syringe	CR 700-20	Hamilton Co., Reno, NV, USA

Instruments for hormone analysis (ELISA)

Homogenizer (stirrer and shafts)	For 1.5 ml tubes	VWR international GmbH, Darmstadt, HE, Germany
Centrifuge	5415R	Eppendorf AG, Hamburg, Germany
Microplate Reader	Multiscan FC	ThermoFisher Scientific Inc., Tow Rivers, WI, USA

Instruments for gene expression analysis (RTQ-PCR)

Homogenizer (stirrer and shafts)	For 1.5 ml tubes	VWR international GmbH, Darmstadt, HE, Germany
Shaker	VXR	IKA® GmbH & Co. KG, Staufen im Breisgau, BW, Germany
Thermal Cycler for RT-PCR	Veritis 96 well Fast	Applied Biosystems GmbH, Darmstadt, HE, Germany
Thermal Cycler for real-time PCR	StepOnePlus	Applied Biosystems GmbH, Darmstadt, HE, Germany

Instruments for DNA isolation

Thermomixer	1.5 ml	Eppendorf AG, Hamburg, Germany
Centrifuge	5415R	Eppendorf AG, Hamburg, Germany
Spectrophotometer	P 360	Implen GmbH, München, Germany

2.4 Electronic consumptive materials

Wires for recording and stimulation electrodes	Insulated Ø 127 μm	BioMedical Instruments, Zöllnitz, TH, Germany
Wires for reference electrodes	Ø 400 μm	Conrad electronic GmbH, Hirschau, BY, Germany
Screws for reference electrodes	M 1.4 mm, K 2.5 mm, L 3.8 mm	Optotechnischer Gerätebau GmbH, Rathenow, BB, Germany
Plastic Sockets	6×4×6 mm or 9×4.5×6 mm	Leibniz Institute of Neurobiology, Magdeburg, ST, Germany

2.5 Chemicals and Reagents

Anisomycin	Sigma Aldrich, St. Louis, MO, USA
5-aza-2'-deoxycitidine (5-aza)	Sigma Aldrich, St. Louis, MO, USA
Cyclofenil	Sigma Aldrich, St. Louis, MO, USA
DEPC water	Roth AG, Karlsruhe, BW, Germany
Dimethylsulfoxid (DMSO)	Sigma Aldrich, St. Louis, MO, USA
100% Ethanol	Roth AG, Karlsruhe, BW, Germany
MasterMix 2×	Applied Biosystems GmbH, Darmstadt, HE, Germany
Pentobarbital sodium salt	Sigma Aldrich, St. Louis, MO, USA
100% 1,2-Propandiol	Sigma Aldrich, St. Louis, MO, USA
* Real-time PCR Assay 20× (primer solutions)	Applied Biosystems GmbH, Darmstadt, HE, Germany
RNase inhibitor	Applied Biosystems GmbH, Darmstadt, HE, Germany
RNA stabilization reagent RNA^{Later}	Qiagen GmbH, Hilden, NW, Germany
Sample Diluents for ELISA	IBL GmbH, Hamburg, Germany
Sodium chloride	Roth AG, Karlsruhe, BW, Germany
Eplerenone	Sigma Aldrich, St. Louis, MO, USA
5 mmol/L Trichostatin A in 100% DMSO	Sigma Aldrich, St. Louis, MO, USA

* Assays: *Hprt1, Esr1, Esr2,Ar, Nr3c1, Nr3c2, Cyp19a1, Bdnf*

2.6 Solutions

48 µg/µl Anisomycin	480 µg Anisomycin 10 µl 100% DMSO
1 µg/µl 5-aza solution	100 µg 5-aza-2'-deoxycitidine 40 µl 1,2-Propandiol 60 µl Distilled water
100 ng/µl Cyclofenil solution	100 µg Cyclofenil 1 ml 100% DMSO
5 mg/ml Nembutal solution	1.25 g Pentobarbital sodium salt 125 ml 1,2-Propandiol 125 ml Distilled water
40% v/v 1,2-Propandiol solution	40 µl 1,2-Propandiol 60 µl Distilled water
0.90% w/v NaCl solution (Saline)	9 g Sodium chloride 1 l Distilled water
50 ng/µl Eplerenone solution	50 µg Eplerenone 1 ml 100% DMSO
Real-time PCR mixture	0.5 µl GAP DH 0.5 µl Assay 20× 5 µl MasterMix 2× 2 µl DEPC water 2 µl cDNA sample

RT-PCR mixture	6 µl 10× TaqMan buffer
	2.4 µl dNTP
	6 µl Primer Hexa Random
	3 µl RNase inhibitor
	3 µl MultiScribe reverse transcription
	9.6 µl DEPC water
	30 µl RNA sample

2.7 Kits

Kits for ELISA

Corticosterone saliva ELISA kit	IBL GmbH, Hamburg, Germany
Testosterone saliva ELISA kit	IBL GmbH, Hamburg, Germany
17β-Estrodiol saliva ELISA kit	IBL GmbH, Hamburg, Germany

Kits for gene expression analysis

High Capacity cDNA Reverse Transcription Kit	Applied Biosystems GmbH, Darmstadt, HE, Germany
RNeasy Plus Mini Kit	Qiagen GmbH, Hilden, NW, Germany

Kits for DNA isolation

GenElute™ Mammalian Genomic DNA Miniprep Kit	Sigma Aldrich, St. Louis, MO, USA

2.8 Softwares

Text management	Microsoft Word 2007

	Adobe® Acrobat 8
Data management	Microsoft Excel 2007
Scientific graphing	GraphPad Prism 5
Statistics	IBM SPSS statistics 20
	GraphPad Prism 5
	Microsoft Excel 2007
Figure management	GraphPad Prism 5
Illustration	Corel Draw 11
Electrophysiology	Pwin, LIN Magdeburg
ELISA	ScanIt Software V 2.5
Video recording	Etho Vision XT (Version 5.0.216)

3. Methods

3.1 Animals and housing conditions

Male Wistar rats (strain Hanover) from the breeding colony, at the Institute of Biology, Otto von Guericke University of Magdeburg, were used for all experiments. Pregnant females were checked for litters daily. At the day of birth (PND0), litters were standardized to five male / five female pups per dam by removal of odd pups. On PND21, male pups were weaned and then housed in sex-matched groups of five male animals per cage (length × depth × height: 59×38×25 cm) until electrophysiological surgery, after which the prepared rats were housed in single-cages (40×25×18 cm) for 1 week. All animals were housed in translucent plastic cages under controlled laboratory conditions [temperature: 21±2 ºC; humidity: 55±5%, 12-hour light/dark circle (light on at 6:00 am)] with free access to food and water. Before weaning, the home cages of all litters were not cleaned. After weaning, comprising cage cleaning was done twice a week.

All experiments were performed in accordance with the European Communities Council Directive of 24th Nov. 1986 (86/609/EEC), and according to the German guidelines for the care and use of animals in laboratory research and the experimental protocols were approved by the ethics committee of the land Saxony-Anhalt. All efforts were made to reduce the number of rats used in this study and their suffering.

3.2 Maternal separation

Litters were randomly assigned to a separation (MS+) group and a non-separation (MS-) group. The litters of MS- group remained undisturbed in their home cages. From PND14 to 16, pups of the MS+ litters group were removed from their dam and transferred in separation boxes with chambers (40×40×40 cm, the space was divided into 4 chambers with plastic crisscross clapboard; one pup per chamber in three boxes) under controlled temperature (31±2 ºC) at 11:00 am. The pups had acoustic and olfactory but not tactile contact to their siblings, but not to their mother or other rats. After 6 hours (at 17:00), the pups were reunited with their dam and siblings in their home cage. Neither food nor water was available during the time of separation.

3.3 Implantation of electrodes

Male rats (10 weeks old, about 300 g) were weighed and anesthetized with Nembutal (Sigma Aldrich, St. Louis, MO, USA) injected intraperitoneally (i.p.) (60 mg/kg bodyweight). The

skulls were fixed in a stereotaxic frame (TSE System, Midland, MI, USA) with an angle (bregma 1 mm higher than lambda). A bipolar stimulation electrode was chronically implanted under stereotaxic control into the right medial perforant path (−6.9 mm AP, 4.1 mm ML, 2.0–2.4 mm DV). A double recording electrode, which consists of a pair of stainless steel wires (125 μm in diameter) coated with Teflon (with a tip length difference of 400 μm), was implanted into the DG (−2.8 mm AP, 1.8 mm ML, 2.8–3.4 mm DV) in the right dorsal hippocampus (fig. 5). The shorter tip was situated in the molecular layer for recording of the excitatory postsynaptic potential (fEPSP), while the longer tip reached the granule cell layer and recorded the population spike amplitude (PSA). Stainless steel screws with silver-coated copper wires were placed on the dura and served as reference electrodes. During preparation, test pulses (0.4 mA) were delivered to optimize electrophysiological signals. Coordinates are based on the atlas of Paxino and Watson (1997). Electrodes and sockets were fixed with dental cement (Heraeus Kulzer GmbH, Hanau, Germany). After preparation, the animals were housed in single-cages and allowed to recover for at least 1 week.

3.4 Electrophysiological recording

During electrophysiological recording, rats were placed in recording boxes (40×40×40 cm, one animal per box) with *ad libitum* access to food and water. The electrodes were connected to a swivel by a flexible cable allowing the animals to move freely. Biphasic constant current pulses (0.1 ms per half wave) were delivered by stimulators (Isolated Pulse Stimulator, Model 2100, A-M Systems, Carlsborg, WA, USA), which were triggered by the software Pwin (Institute of Neurobiology, Magdeburg). The evoked field potentials were amplified (100×; differential AC amplifier model 1700, Science Products, Hochheim, Germany), transformed by an analog/digital interface (CED 1401+; Cambridge Electronic Design, Cambridge, UK) and stored on a computer. At day 1, an input/output curve was measured, in which the slope of the fEPSP and the amplitude of the population spike were measured with the ascending stimulation intensity (maximal 0.8 mA). The stimulation intensities that evoke 60% of the maximum fEPSP (in the range of 0.05–0.4 mA) and as 40% of the maximum PSA (0.05–0.8 mA), respectively (no more than 0.4 mA) was adopted to establish a baseline.

At day 2, after registering of a stable baseline for 1 h (mean value of 5 stimuli with 10 s inter-pulse interval), LTP was triggered by application of a tetanic stimulation. Two tetanization modes were applied in the present study. One is strong tetanus, which comprises 10 bursts of 15 pulses of 200 Hz with 0.1 ms duration of each stimulus. Strong tetanus has been proved to be able to induce late-LTP directly. The other mode is weak tetanus, which is composed of 3

bursts of 15 pulses of 200 Hz with 0.1 ms duration of each stimulus and 10 s inter-burst interval. Weak tetanus alone can only induce early LTP. Both tetanization modes applied the same stimulus intensity as used for the PSA baseline.

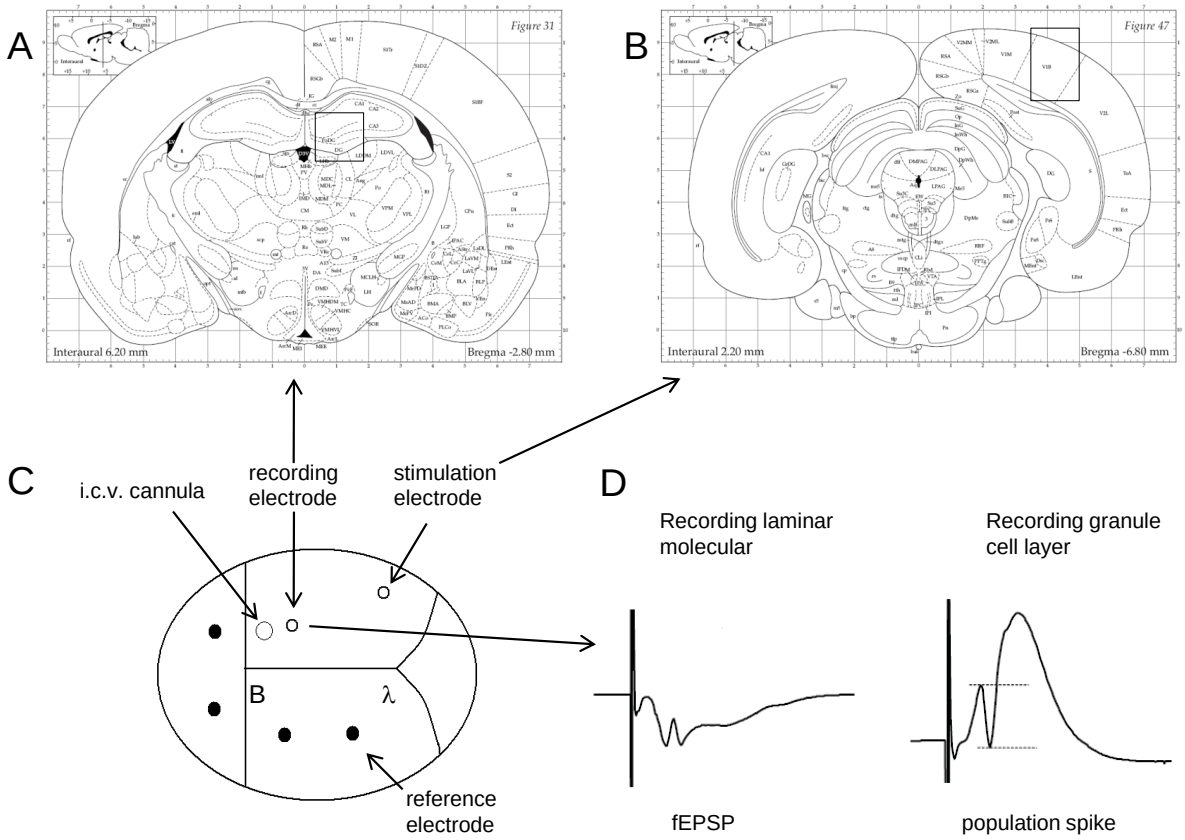

Figure 5. Implantation sites of electrodes and cannulas. (A) and (B) show schematic views of coronal brain slides at AP direction of electrode implantation (atlas by Paxinos and Watson, 1998). (A) Recording electrodes with a longer tip and a shorter tip were implanted into the dentate gyrus. (B) A bipolar Stimulation electrode was implanted into right medial perforant path. (C) A scheme of the scul pointing out positioning of references (small points), i.c.v. cannula (large open circle) and electrodes (small open circles) in relation of bregma (B) and lambda (λ). (D) The left signal is fEPSP recorded by the shorter tip of recording electrode located in the dentritic layer; the right signal shows population spike (PS) recorded by the longer tip located closed to the cell body.

A part of the animals stimulated with weak tetanus was stressed by elevated platform (EPF). Animals were randomly assigned to two conditions: two animals remained in the recording boxes, while two littermates were disconnected from the cables in the recording boxes immediately after tetanus and exposed to EPF-stress for 10 min. The rats were placed on a quadratic platform (20×20 cm) 150 cm above ground. Afterwards, the LTP of all animals was measured by test stimuli (mean value of 5 stimuli with 10 s inter-pulse interval) with 15 min interval. During recording, fEPSP and PSA were evoked and recorded by turns, so that two

fEPSP and two PSA were measured every hour. In order to record the late phase of LTP, measurements proceeded for the next 6 h. Then, a 24 h value was obtained at day 3.

Different groups of rats were sacrificed 15 min, 1 h and 24 h respectively after tetanus/stress. Tetanization/stress exposition always took place between 10:00 and 11:00 am to consider the diurnal rhythm of hormone release and gene expression. After one-week recovery, in few animals, only the PSA or fEPSP was measurable. Therefore, the sample sizes between the two measures are not equal in some cases. When only the fEPSP was measurable, tetanization took place with an intensity of 0.4 mA.

3.5 Drugs and infusions

Pharmacological treatment was performed intracerebroventricularly (i.c.v.) at young adult rats during electrophysiological recording. Pharmacologically treated animals were, in addition to the electrodes, equipped with cannulas (700 μm in outer diameter, reaching 4 mm into the brain) implanted into the right lateral ventricle (-0.8 mm AP from bregma, 1.5 mm ML from midline; fig. 5). During electrophysiological recording, rats were randomly assigned into two groups, treated either with drugs (5-aza-2'-deoxycitidin, trichostatin A, cyclofenil, eplerenone or anisomycin) or with corresponding vehicle solutions. All drugs and vehicles were infused at a rate of 1 μl/min and injectors were left in the cannula for at least 5 min after infusion to allow the substances to diffuse properly. The used concentrations of all the drugs have been checked not to affect baseline PSA and fEPSP.

For the enzyme inhibitors (5-aza-2'-deoxycitidine and trichostatin A), the i.c.v. infusion was performed 2 h before tetanus/EPF-stress. 5-aza-2'-deoxycitidine (5-aza) was the inhibitor of DNMT. This drug was dissolved in 40% propandiol (Sigma) with a final concentration of 1 μg/μl, while 40% propandiol was used as vehicle. A total volume of 5 μl drug solution or vehicle was injected. TSA (the inhibitor against HDAC) dissolved in 100% DMSO (Sigma) and 100% DMSO used as vehicle were infused with a total volume of 3 μl.

Different to the inhibitors of enzymes, the antagonists of ERβ and MR receptors (cyclofenil and eplerenone) were injected 10 min before tetanus/stress. Cyclofenil (Sigma), the selective antagonist of ERβ, was dissolved in 100% DMSO at 60 ºC (after solution stored at 4 ºC). A total volume of 5 μl cyclofenil solution or vehicle was infused. The same as cyclofenil, eplerenone (sigma), the mineralocorticoid receptor antagonist, was also dissolved in 100% DMSO (after solution stored at 4 ºC). The injection was performed with a total volume of 3 μl.

Anisomycin (Sigma), a reversible protein-synthesis-inhibitor was also applied by i.c.v. infusion 2 h before tetanus/EPF-stress. This drug was dissolved in 100% DMSO with a final concentration of 48 µg/µl. A total volume of 5 µl drug solution or vehicle (100% DMSO) was infused per i.c.v.

3.6 Behavioral tests

The elevated plus maze is a widely used method for assessing anxiety responses of rodents (Pellow et al., 1985). The test/retest (trial 1 / trial 2) protocol was employed in the present study (File et al., 1990). Our apparatus of elevated plus maze was 100 cm high and consisted of four arms (10 cm x 50 cm each) made of grey plastic. Two (opposite of each other) of them were equipped with grey plastic walls (30 cm high). From the center arena (10 cm x 10cm), the rats started to explore the maze. Each 5 min trial was videotaped and subsequently analyzed by the tracking program Etho Vision XT (Version 5.0.216). Assessment included the time spent in open arms, closed arms and center region of the maze, as well as the velocity and path length throughout every single trial. Rats were handled exactly as during the electrophysiological experiments, including a one-week single keeping time.

The animals were brought from the facility room to the testing room 2 h prior to testing. Rats that were taken out from the cages were marked with a permanent marker (for video tracking) at head and neck regions. Then, they were set on the center arena. After the trial (5 min), the rats were set back into their cages and brought back to the facility room. Conducted were two trials with a 24 h interval. Fifteen min after the second trial, the animals were decapitated, while their trunk blood was collected and their right hippocampi were dissected out from the brain. Samples were treated identically as during the electrophysiological studies.

3.7 Tissue sampling and hormone assaying

After electrophysiological recording or behavioral tests, animals were decapitated, their trunk blood was collected and the right hippocampi were rapidly dissected on ice. The tissues were homogenized with electronic pestles and diluted with sample diluent (IBL Hamburg, REF KLZZ731) to reach a final volume of 25 µl/mg tissue weight. Blood samples were incubated at room temperature for at least 30 min. Then, all samples were centrifuged (10 min, 13200 rpm). The supernatant was aliquotized and stored at −20 °C.

Before hormone assaying, tissue and blood samples were diluted 1:2 and 1:10 respectively with sample diluents (IBL Hamburg, REF KLZZ731). Then, an enzyme-linked immuno-sorbent assay (ELISA) was performed, according to the manufacturer’s instructions. OD

values were measured with a microplate reader (Multiscan FC, Thermo Scientific) at 450 nm. The ScanIt Software V 2.5 (Thermo Scientific) was used to calculate the sample values of hormones by the standard four-parameter logistics plot.

For the corticosterone kit [Corticosterone ELISA (RE52211) by IBL Hamburg], the minimum detectable dose of corticosterone was 1.63 nmol/l and intra-assay and inter-assay coefficients of variation were 5.0 and 7.0%, respectively. For the testosterone assay [Testosterone Saliva ELISA (RE52631) by IBL Hamburg], the limit of detection (LOD) was 2.0 pg/ml and intra-assay and inter-assay coefficients of variation were 8.2 and 5.5%, respectively. The estradiol kit [17β-Estradiol Saliva ELISA (RE52641) by IBL Hamburg] had a LOD of 0.4 pg/ml. Intra-assay and inter-assay coefficients of variation was maximally 9.9 and 11.1%, respectively.

3.8 Gene expression analysis

1 h after tetanus/stress, animals were decapitated and the right hippocampi were rapidly dissected on ice, added with mRNA stabilizing agent (RNA^{Later}, Qiagen) and stored at -80 °C. After RNA extraction (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Darmstadt) and reverse transcription polymerase chain reaction (RT-PCR) were done, gene expression analysis (real-time PCR) was performed then with a StepOnePlus machine from Applied Biosystems. TaqMan® Gene Expression Assays with the classification "m" were used exclusively, which primers span an exon junction and detect only genomic DNA (Taqman® Universal PCR Master Mix). Assays were done in triplicate.

The PCR cycling conditions:

RT-PCR: 25 °C 10 min; 37 °C 120 min; 85 °C 5 min; 4 °C ∞

Real-time PCR: 50 °C 2 min; 95 °C 10 min; 45 × (95 °C 15 s; 60 °C 1 min); 4 °C ∞

The comparative cycle threshold (C_T value) method was used to calculate differences in gene expression between samples (Livak and Schmittgen, 2001). The comparative cycle threshold method, also referred to as $2^{-\Delta\Delta C_T}$, is a widely used method to present relative gene expression, which presents the RT-PCR data of the genes of interest relative to some calibrator or internal control gene (Schmittgen and Livak, 2008). An endogenous control gene and a reference gene are used in this method to calculate the relative expression level of the gene of interest. The following formula is applied:

$$\Delta\Delta C_T = (C_{T\ \text{gene of interest}} - C_{T\ \text{endogenous control}}) - (C_{T\ \text{reference gene}} - C_{T\ \text{endogenous control}}).$$

Then, $2^{-\Delta\Delta C_T}$ will reflect the relative expression level of the gene of interest, with which the statistical analyses can be performed to compare the mRNA level of an interesting between groups.

As the endogenous control for real-time PCR, we used Rat GAPDH (Vic®/MGB Probe), while *Hprt1* served as the reference gene. All reagents were from Applied Biosystems. Relative quantification was used to give expression levels. The relative expressions of estrogen receptor (ERα) gene (*Esr1*) and ERβ gene (*Esr2*), the androgen receptor (AR) gene (*Ar*), the mineralocorticoid receptor (MR) gene (*Nr3c2*), the glucocorticoid receptor (GR) gene (*Nr3c1*), *Cyp19* gene as well as *Bdnf* gene, the gene for the brain-derived neurotrophic factor (BDNF), have been measured. *Cyp19a1* gene encodes the enzyme aromatase, which converts testosterone into estradiol. Since interactions between BDNF and estrogen in the regulation of hippocampal plasticity (Scharfman and MacLusky, 2006) have been reported, we measured also BDNF gene expression.

3.9 DNA methylation assay

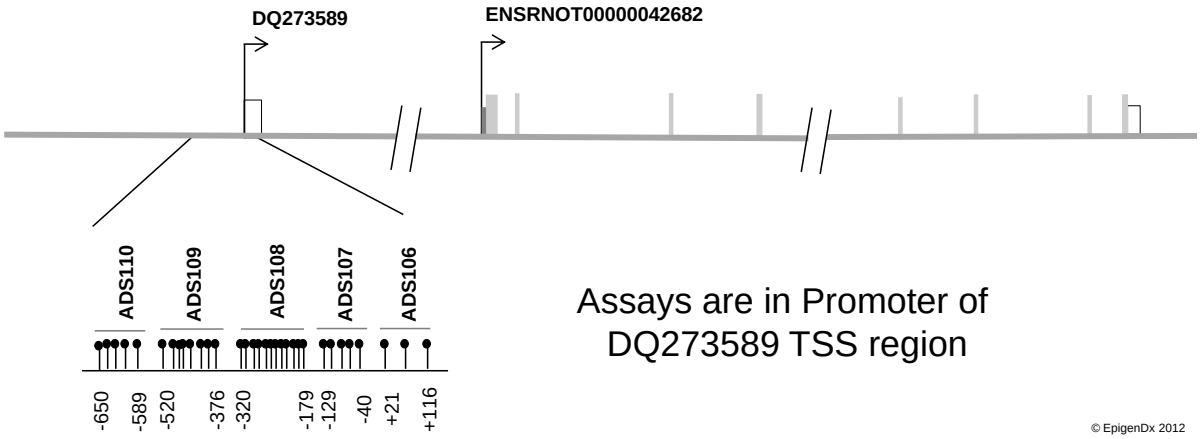

Figure 6. The region of methylation analysis along the ERβ gene (*Esr2*) promoter consists of 33 CpG dinucleotides ranging from -655 to +116 in relation to the transcription start site (+1) DQ273589. These CpG sites were artificially divided into 5 clusters (ADS 106 to ADS 110) according to their locations. TSS: transcription start site. Due to technical problems, site 8 could not be analyzed properly.

The same as gene expression analysis, animals from MS+ and MS- groups were sacrificed and decapitated 1 h after EPF-stress or under basal condition (without EPF-stress). Right hippocampus was dissected and put into liquid nitrogen immediately. Tissues were stored at -

80 °C until use. Genomic DNA was isolated with GenElute^{TM} Mammalian Genomic DNA Miniprep kit (Sigma Aldrich, St. Louis, MO, USA) according to manufacturer's direction. The DNA concentration of all samples was uniformed to 20 ng/μl. Then, the samples were stored at -20 °C.

Methylation assays for *Esr2* gene promoter, including bisulfite conversion, were designed and performed by EpigenDX (Worcester, MA, USA). All primer sequences are property and owned by EpigenDX. These assays covered 33 CpG sites (due to technical malfunction, the data of CpG site 8 were not reliable, so the presented data included only 32 sites) within the promoter region DQ2735589 TSS (fig. 6). The determination of methylation status of CpG sequences was carried out by pyrosequencing for allele quantification (PSQ H96A; Biotage, Uppsala, Sweden). The detailed information on the sequences of the CpG islands and the procedure of DNA methylation assay are presented in the appendix.

3.10 Statistics

The general linear model (GLM) for repeated measures was used for group comparisons of overall differences in LTP time course, while Students t-test for independent samples was used to evaluate LTP differences of single time points between groups.

Overall comparisons in hormone and gene expression (mRNA) level were at first estimated with multivariate GLM analysis, because possible dependencies between variables could not be ruled out. One-way ANOVA and Tukey post-hoc tests were used to identify significant differences between single groups. Two-group comparisons were done with the student t-test. Curve estimation was performed to analyze the correlation between the expression levels of two genes.

The analyzed data for DNA methylation assays were presented as percent methylation in every single CpG site and compared between groups by student t-test (Schwarz et al., 2010).

The behavioral data of EPM was analyzed by t-test with Bonferroni-Holmes correction between MS+ and MS- rats, and by paired-sample t-test between trials. One-sample t-test was applied to compare the duration of a rat group in an area in the EPM with the random distribution of this area.

All tests were two-tailed and the level of significance was set at $p \leq 0.05$.

4. Results

4.1 The effects of MS on hippocampal LTP

4.1.1 MS did not affect the basic electrophysiological properties of the hippocampal DG granule neurons

At first, we tested whether the early-life experience affected the body weight in adulthood. The mean body weight of adult rats with MS was slightly but significantly lower (301.0 +/- 4.35 g) than control animals (313.5 +/- 3.57 g, p = 0.033).

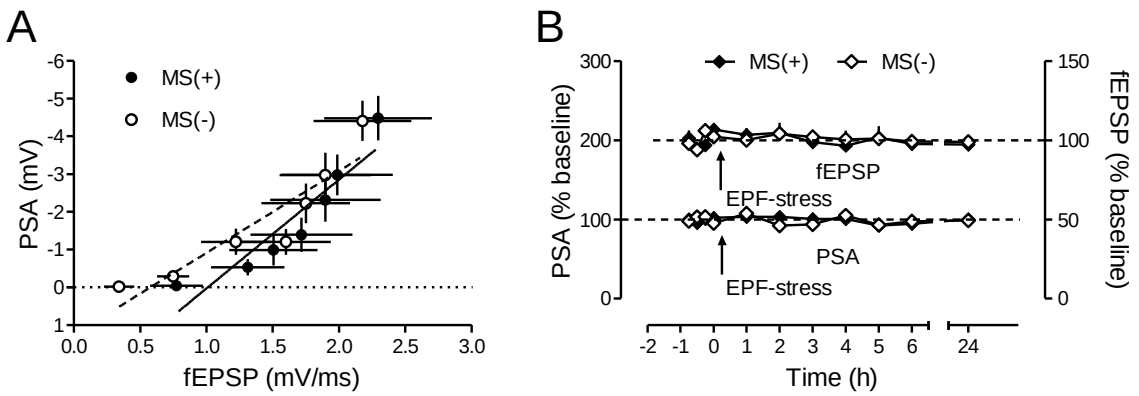

Figure 7. Maternal separation did not alter basic electrophysiological properties in the hippocampal dentate gyrus. (A) Granule cell excitability as indicated by the regression line slopes of ES-plots as a function of stimulation intensity, was not different between MS+ (n = 8) and control (MS-) rats (n = 8). (B) Baseline synaptic transmission during stress experiments in MS+ (n = 8) and MS- rats (n = 8) was similar between groups. Given are the means and SEM.

Then, we focused on the effects of MS on the basic electrophysiological properties of the DG. MS did not affect the basic electrophysiological properties of granule cells in the DG. No significant differences in cell excitability were found between maternally separated and non-separated rats. The ES-plots (the mean fEPSP values against PSA values as a function of stimulus intensity) revealed similar slopes of the regression lines for both groups (p = 0.312) (fig. 7 A). Moreover, no difference in basic synaptic transmission could be detected between MS+ and MS- rats over the time course of the experiment (fEPSP: p = 0.823; PSA: p = 0.721) (fig. 7 B).

When LTP was triggered in the DG, the high frequency stimulation (tetanus) without post-tetanic stress (fig. 8 A and B) induced statistically same time courses of LTP (fEPSP: p = 0.515; PSA: p = 0.425) in both MS+ and MS- rats.

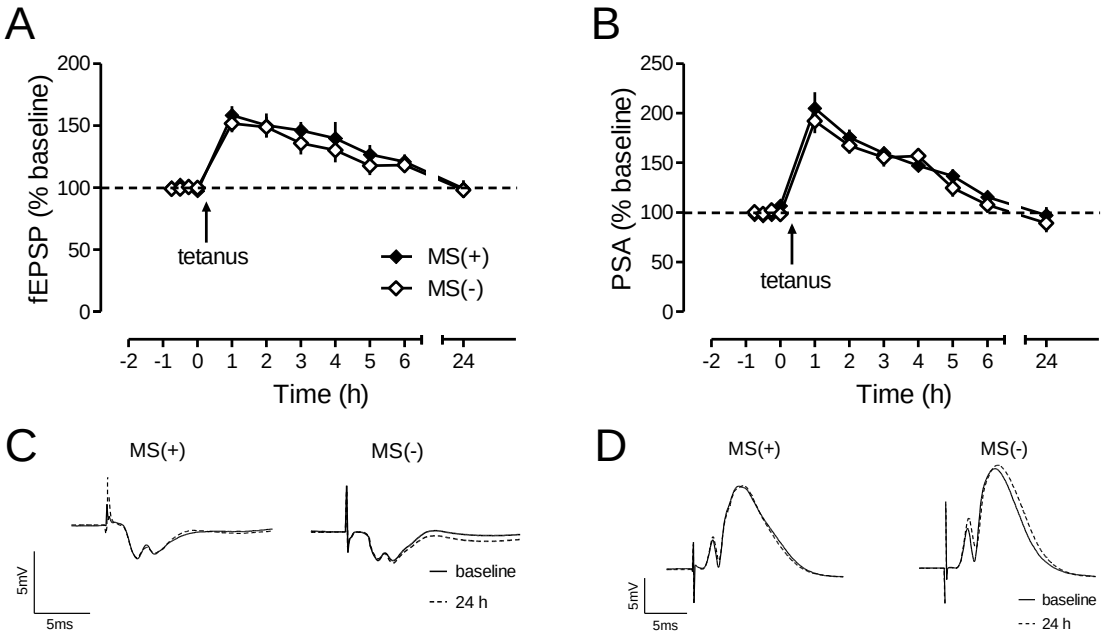

Figure 8. Maternal separation did not alter the time course of early LTP. (A, B) Week tetanus induced the same time courses of LTP in both MS+ and MS- rats. (A) The time courses of fEPSP-LTP (MS+: n = 6, MS-: n = 7). (B) The time courses of PSA-LTP (MS+: n = 8, MS-: n = 6). Representative analog traces of the fEPSP and the PSA of MS+ and MS- rats under no-stress condition are depicted in (C) and (D). Given are the means and SEM.

4.1.2 MS blocked emotional LTP reinforcement

In contrast to early LTP, late-LTP induced by post-tetanic stress was significantly impaired by MS. Exposing animals to a 10-min elevated-platform-stress (EPF-stress) immediately after tetanus (fig. 9) triggered significant differences in the time courses of LTP between MS- and MS+ rats in both the fEPSP ($F_{3, 27} = 4.742$, $p = 0.009$) and the PSA ($F_{3, 27} = 11.653$, $p < 0.001$). Post hoc tests identified that the PSA-LTP of the stressed MS- rats was significantly reinforced, compared to non-stressed MS- rats ($p < 0.001$) and stressed MS+ rats ($p = 0.001$), whereas EPF-stress did not prolong the time course of PSA-LTP (fig. 9 B) in MS+ rats ($p = 0.238$). The same effect could be observed in the fEPSP-LTP (fig. 9 A). Here, LTP of the stressed MS- rats was significantly reinforced by EPF-stress, compared to either of the non-stressed rats ($p = 0.009$) and stressed MS+ rats ($p = 0.001$), whereas EPF-stress did not alter the time courses of fEPSP-LTP in MS+ rats ($P = 0.251$).

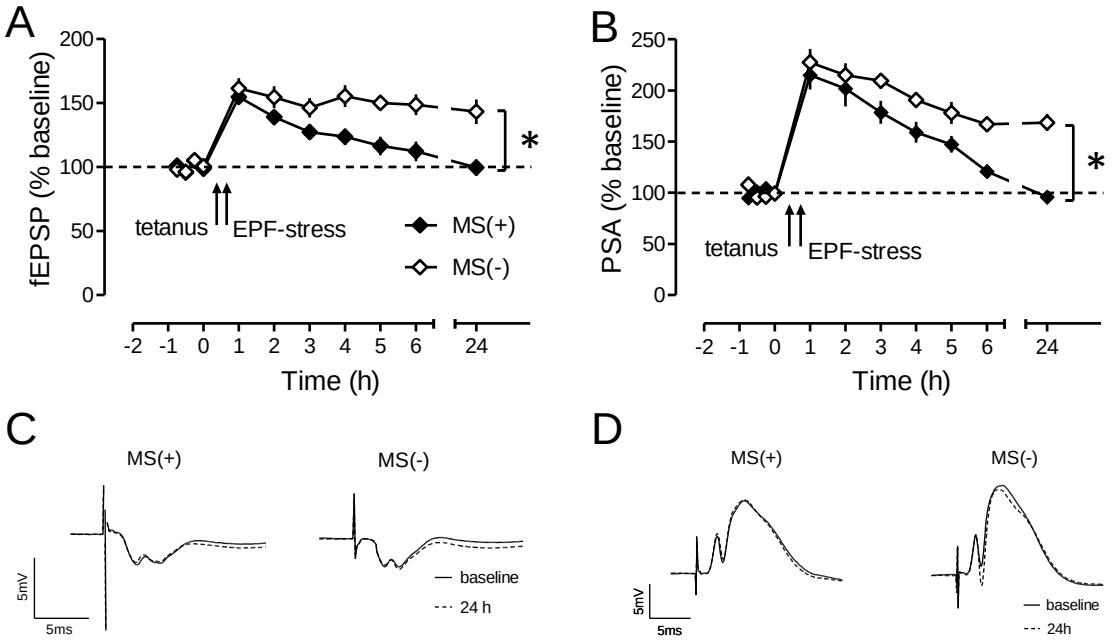

Figure 9. The LTP-reinforcement induced by a 10-min EPF-stress was inhibited by maternal separation. (A, B) LTP of fEPSP (A, MS+: n = 9, MS-: n = 8) and PSA-LTP (B, MS+: n = 9, MS-: n = 9) were significantly reinforced by an EPF-stress immediately after week tetanus, in MS- rats but not in MS+ rats. Representative analog traces of the fEPSP and the PSA of MS+ and MS- rats under stress condition are depicted in (C) and (D). Given are the means and SEM. Asterisks indicate significant differences.

4.1.3 The emotional LTP-reinforcement in control rats depends on protein-synthesis

Late-LTP has been proved to be protein-synthesis-dependent, as anisomycin, a reverse translation inhibitor, can block LTP beyond 4 – 6 h after tetanus (Krug et al., 1984; Frey et al., 1988; Reymann et al., 1988a). Even the late-LTP induced by post-tetanic swim-stress depends on protein-synthesis (Korz and Frey, 2003). But we still want to verify whether the EPF-stress-induced LTP-reinforcement observed in the control (MS-) rats in the present study is also protein-synthesis-dependent. Thus, the i.c.v. infusion with anisomycin was performed in the MS- rats 2 h before tetanus/EPF-stress.

Then, it was found that the application of anisomycin significantly prevented LTP-reinforcement (fig. 10). The rats treated with anisomycin (n = 6) showed a significantly impaired fEPSP-LTP ($p = 0.034$; fig. 10 A) in comparison to the vehicle controls (n = 8). Although PSA-LTP (fig. 10 B) did not show significant difference between the groups (anisomycin: n = 6; vehicle: n = 8) over the whole time course ($p = 0.052$), it was significantly impaired in the phase of late-LTP (4–24 h) by the blockade of translation (p =

0.002). These results prove that the LTP-reinforcement induced by post-tetanic EPF-stress also depends on protein-synthesis.

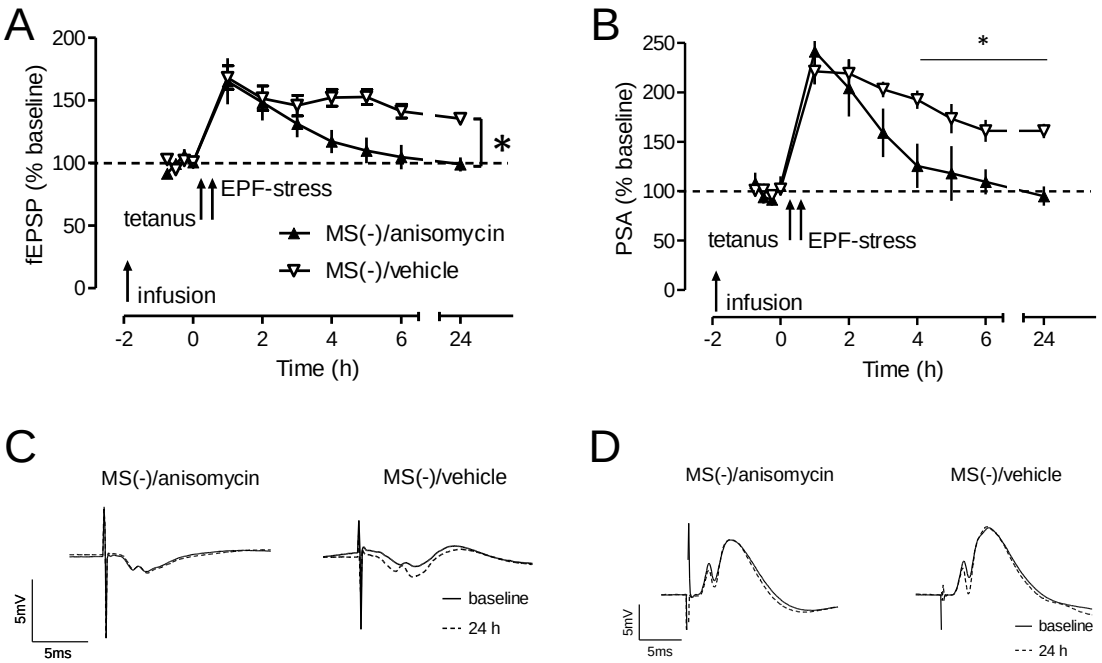

Figure 10. The application of the reverse translation inhibitor anisomycin prevented the EPF-stress-induced LTP-reinforcement. (A) The fEPSP-LTP was severely impaired by the i.c.v. infusion of anisomycin (n = 6) as compared to vehicle controls (n = 8). (B) PSA-LTP was significantly impaired in the phase of late-LTP (4–24 h) in anisomycin-treated rats (n = 6) as compared to vehicle controls (n = 8). Representative analog traces of fEPSP and PSA of drug and vehicle treated MS+ rats are given in (C) and (D). The horizontal bar shows the difference between anisomycin-treated group and vehicle group in late phase of LTP. The vertical bars showed the differences of the whole time-course of LTP. Given are the means and SEM. Asterisks indicate significant differences.

4.1.4 MS did not affect electrically induced late-LTP

The above-mentioned results verify an inhibitory effect of MS on stress-induced late-LTP. However, it was still unknown whether this inhibitory effect of MS was attributed to a damaged ability of late-LTP formation or a repressed responsiveness to stress. Thus, we compared the late-LTP induced by strong tetanus between MS+ and MS- rats. Then, it was found that late-LTP could be induced normally in both groups (fig. 11). Strong tetanus triggered statistically same time courses of LTP (fEPSP: $p = 0.664$; PSA: $p = 0.662$) in both MS+ and MS- rats. This result indicates that MS did not affect the ability of late-LTP formation and suggests a defect of hippocampal stress-responsiveness in early-stressed rats.

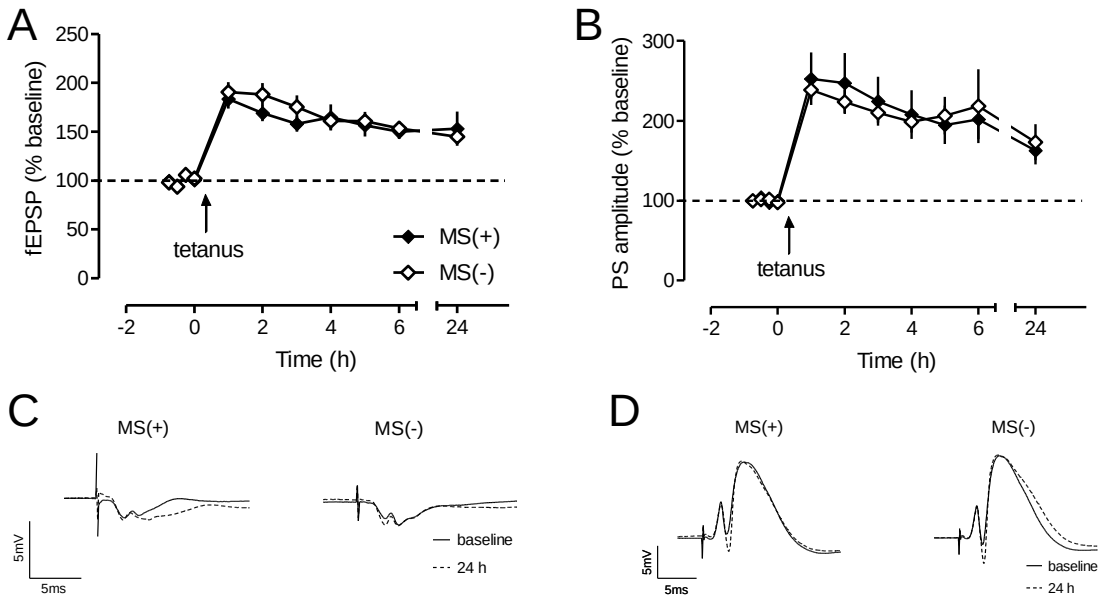

Figure 11. Maternal separation did not alter the time course of late-LTP induced by an electrical strong tetanus. (A, B) Strong tetanus induced late-LTP in both MS+ and MS- rats. No significant difference between MS+ and MS- rats was detectable in the time courses of strong-tetanus-induced late-LTP. (A) The time courses of fEPSP-LTP (MS+: n = 8, MS-: n = 8). (B) The time courses of PSA-LTP (MS+: n = 8, MS-: n = 7). Representative analog traces of the fEPSP and the PSA of MS+ and MS- rats under no-stress condition are depicted in (C) and (D). Given are the means and SEM.

4.2 The neurobiological effects of MS on the hippocampus

Steroid hormones and their receptors are found to play critical roles in hippocampal plasticity in response to stress (McEwen, 1999, 2010). In order to reveal the mechanisms underlying the above-mentioned MS-induced defects in stress-induced LTP-reinforcement in the hippocampus, we compared hippocampal concentrations of corticosterone, testosterone and 17β-estradiol (for each group and each hormone: n = 8), as well as the hippocampal mRNA level of MR, GR, AR, ERα, ERβ, aromatase and BDNF 1 h after tetanus/EPF-stress between MS+ and MS- rats (for each group and each gene: n = 8). Since corticosterone reacts rapidly to acute stress, corticosterone level was also measured 15 min after tetanus/EPF-stress.

4.2.1 MS altered hippcampal level of steroid hormones

Multivariate GLM analysis revealed an effect of adult stress (EPF-stress) on hippocampal levels of corticosterone (both 15 min and 1 h after tetanus/stress) and testosterone but not 17β-estradiol, whereas early-life stress (MS) had no effect on all three hormones. No interaction between early-life stress and adult stress was detected in the hippocampal

concentrations of all three hormones at all time points (table 1; fig. 12 A, B, C). The ANOVA revealed significant differences in hippocampal testosterone concentrations ($F_{3, 28} = 3.739$, $p = 0.022$) between groups. EPF-stress elicited a significant increase in testosterone (fig. 12 C) in MS+ rats 1 h after tetanus/EPF-stress ($p = 0.005$), while no such increase was found in MS- rats ($p = 0.492$).

Table 1: Multivariate GLM analysis for the concentrations of three hormones in the hippocampus [AS: adult stress (EPF-stress); ELS: early-life stress (MS); AS * ELS: interaction; df: 1, error 28, each]. Significance is indicated by bold numbers.

Factor	Variable	F	Sig.
AS	Corticosterone 15 min	43.105	**0.000**
	Corticosterone 1 h	6.863	**0.014**
	17β-estradiol	0.776	0.386
	Testosterone	7.100	**0.013**
ELS	Corticosterone 15 min	0.017	0.897
	Corticosterone 1 h	0.000	0.992
	17β-estradiol	0.041	0.841
	Testosterone	1.291	0.265
AS * ELS	Corticosterone 15 min	0.956	0.337
	Corticosterone 1 h	0.076	0.785
	17β-estradiol	0.005	0.942
	Testosterone	2.826	0.104

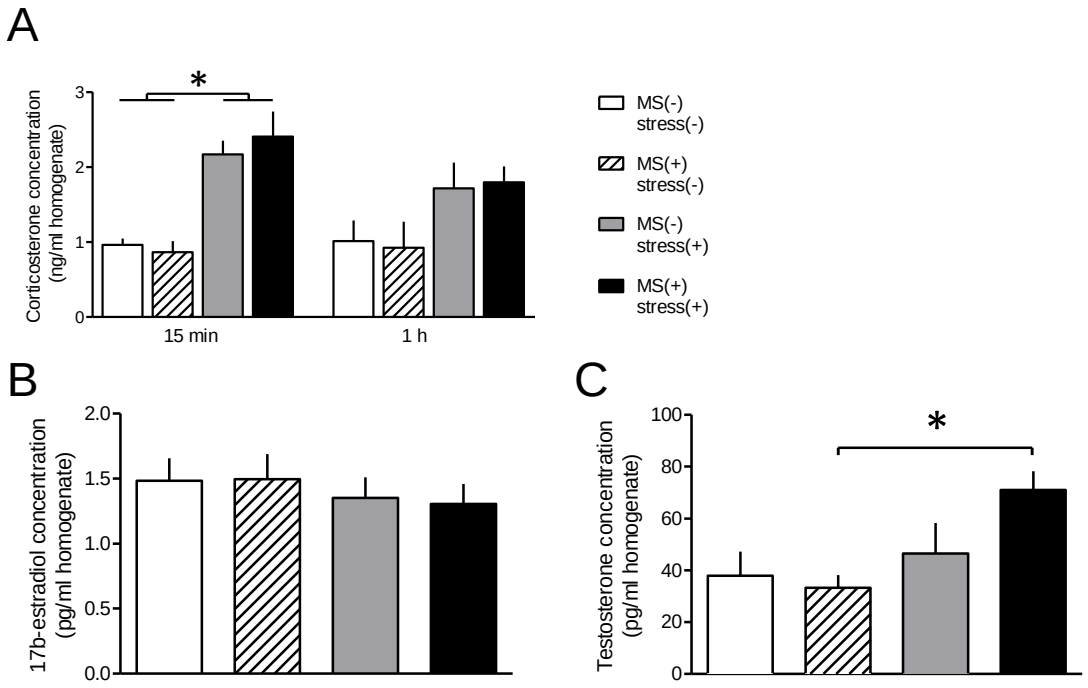

Figure 12. MS+ and MS- rats differed in their responses to post-tetanic stress in hippocampal steroid hormone concentrations. (A) Corticosterone concentrations were significantly higher in both MS+ and MS- rats 15 min post-stress with no difference between groups (n = 8, each). No difference was detected between groups 1 h after tetanus/EPF-stress. (B) No change of hippocampal 17β-estradiol concentrations could be detected (n = 8, each) 1 h after tetanus/EPF-stress. (C) EPF-stress elevated testosterone concentration in MS+ rats, but not in MS- rats (n = 8, each) 1 h after tetanus/EPF-stress. Given are the means and SEM. Asterisks indicate significant differences.

4.2.2 MS altered gene expression levels of steroid hormone receptors in the hippocampus

With regard to gene expression, adult stress significantly affected the hippocampal mRNA level of ERα, ERβ, MR and GR. Early-life stress affected only MR expression and with a tendency also ERβ, whereas an interaction between early-life stress and adult stress was found in MR and ERβ expression levels (table 2; fig. 13 A, C). Multivariate GLM analysis of groups established a difference between ERβ ($F_{3, 28} = 3.31$, $p = 0.034$; fig 13 E), MR ($F_{3, 28} = 7.311$, $p = 0.001$, fig. 13 A) and GR ($F_{3, 28} = 3.98$, $p = 0.018$; fig. 13 A) mRNA-expression 1 h after tetanus/stress. Tukey post-hoc tests identified that EPF-stress induced an increase of the hippocampal ERβ in control ($p = 0.022$), but not in early stressed rats ($p = 0.799$). MR mRNA levels in MS- rats 1 h after tetanus/stress was elevated as compared to all other groups ($p < 0.05$, each), while no difference could be observed between MS+ groups ($p > 0.1$, each). GR mRNA levels were upregulated in the adult-stressed MS+ rats only with a tendency to

significance as compared to unstressed animals (p = 0.076). There were no significant differences between groups in the gene expression of aromatase and BDNF (each, p > 0.05).

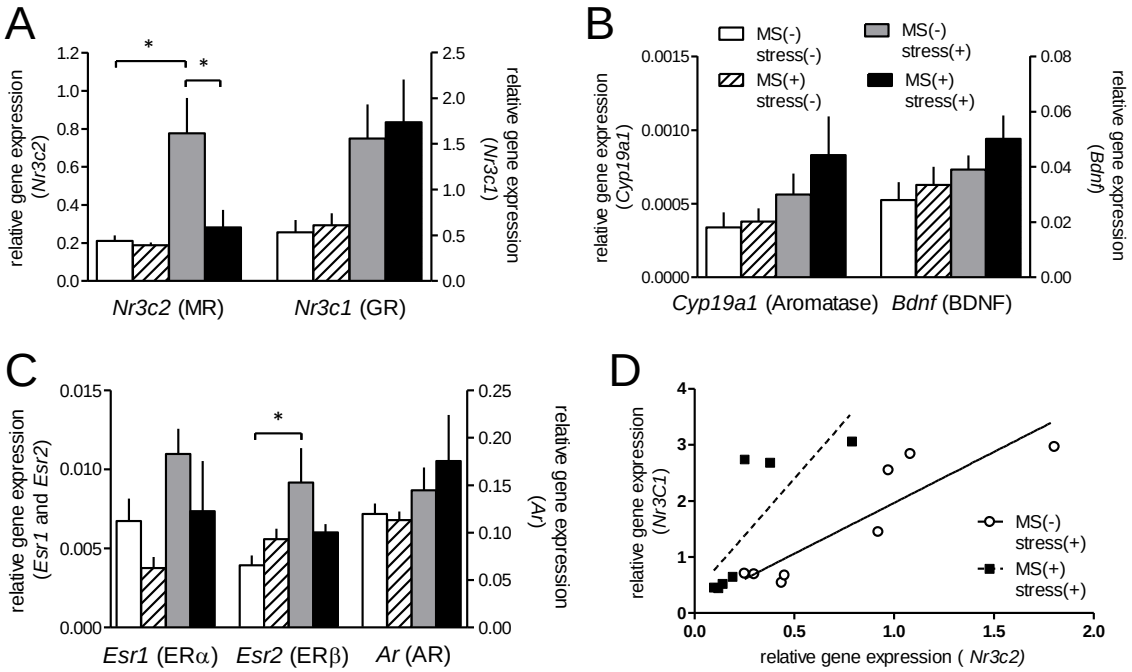

Figure 13. MS+ and MS- rats differed in their responses to post-tetanic stress in hippocampal gene expression of steroid hormone receptors. The mRNA level of MR (A) and ERβ (C) was upregulated by post-tetanic stress in MS- rats but not in MS+ rats, 1 h after tetanus/EPF-stress. GR expression was increased in MS+ rats only with a statistical tendency without difference between groups (A, n = 8, each). The other genes showed no difference in expression between groups. (D) EPF-stress induced a linear correlation between hippocampal gene expression of MR and GR in both MS+ and MS- rats. The slopes of MR/GR correlation lines were not significantly different between MS+ and MS- rats, whereas the intercept of the line of MS+ group was significantly lower than MS- group. Given are the means and SEM. Asterisks indicate significant differences.

According to the findings on MR and ERβ gene expression, the correlations between the expression level of these two genes and the other tested genes were analyzed with curve estimation. The most important finding is that MR and GR gene expression showed linear correlation in both MS+ (p = 0.032) and MS- groups (p = 0.002) after EPF-stress (fig. 13 D), whereas there was no significant linear correlation (but with tendency) between expression levels of these two genes without EPF-stress (MS+: p = 0.092; MS-: p = 0.065). It suggests that acute stress can make the hippocampal gene expression of MR and GR positively correlated by the activation of relevant transcription factors. The slopes of MR/GR correlation

lines were not significantly different between MS+ and MS- rats (p = 0.104), whereas the intercept of the line of MS+ group was significantly lower than MS- group (p = 0.028).

Table 2: Multivariate GLM analysis for $2^{-\Delta\Delta C_T}$ values (relative gene expression) for the 7 genes of interest [AS: adult stress (EPF-stress); ELS: early-life stress (MS); AS * ELS: interaction; df = 1, error = 28, each). Significance is indicated by bold numbers.

Factor	Variable	F	Sig.
AS	ERα (*Esr1*)	4.828	**0.036**
	ERβ (*Esr2*)	5.894	**0.022**
	AR (*Ar*)	2.433	0.130
	MR (*Nr3c2*)	12.407	**0.001**
	GR (*Nr3c1*)	11.746	**0.002**
	Aromatase (*Cyp19a1*)	4.211	**0.050**
	BDNF (*Bdnf*)	4.173	0.051
ELS	ERα (*Esr1*)	3.449	0.074
	ERβ (*Esr2*)	0.452	0.507
	AR (*Ar*)	0.184	0.671
	MR (*Nr3c2*)	7.802	**0.009**
	GR (*Nr3c1*)	0.170	0.683
	Aromatase (*Cyp19a1*)	0.874	0.358
	BDNF (*Bdnf*)	1.472	0.235
AS * ELS	ERα (*Esr1*)	0.032	0.859
	ERβ (*Esr2*)	4.265	**0.048**
	AR (*Ar*)	0.436	0.515
	MR (*Nr3c2*)	6.604	**0.016**
	GR (*Nr3c1*)	0.025	0.874
	Aromatase (*Cyp19a1*)	0.489	0.490
	BDNF (*Bdnf*)	0.172	0.682

4.3 The roles of ERβ and MR in stress-induced LTP-reinforcement

4.3.1 The respective application of ERβ and MR antagonists impaired emotional LTP-reinforcement

Although the correlation between LTP-reinforcement and the upregulation of the hippocampal ERβ gene expression suggests the critical role of ERβ in emotional LTP-reinforcement, we still applied the selective ERβ antagonist (cyclofenil) to confirm the function of ERβ in control (MS-) rats. Then, it was found that i.c.v. infusion of cyclofenil 10 min. before tetanus/EPF-stress (fig. 14 A) did not alter the time course of fEPSP-LTP ($p = 0.235$). However, PSA-LTP (fig. 14 B) was significantly impaired in the phase of late-LTP (4–24 h) by the blockade of ERβ activity ($p = 0.041$), though there was no significant differences between groups over the whole time course of LTP ($p = 0.235$).

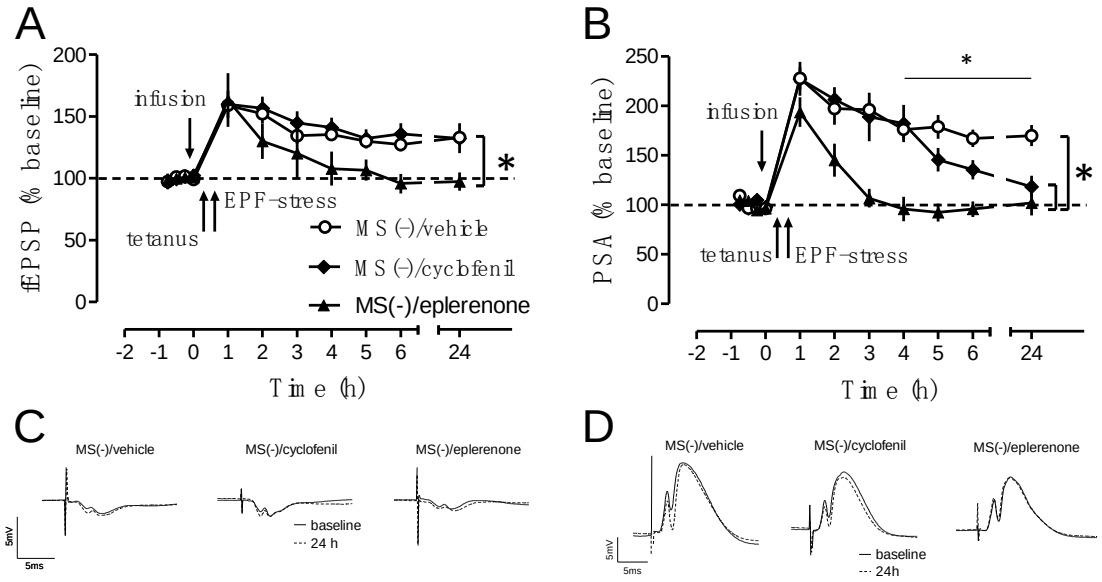

Figure 14. The application of ERβ and MR antagonists altered the time courses of LTP. (A) No significant difference in fEPSP-LTP was detected between rats treated with ERβ antagonist cyclofenil (n = 8) and vehicle controls (n = 8). In contrast, fEPSP-LTP was severely impaired by MR antagonist eplerenone (n = 7). (B) PSA-LTP was significantly impaired in the phase of late-LTP (4–24 h) in cyclofenil-treated rats (n = 9) as compared to vehicle controls (n = 8), while the application of eplerenone impaired the whole time course of LTP (n = 7). Representative analog traces of fEPSP and PSA of drug and vehicle treated MS+ rats are given in (C) and (D). The horizontal bar shows the difference between cyclofenil-treated group and vehicle group in late phase of LTP. The vertical bars showed the differences of the whole time-course of LTP. Given are the means and SEM. Asterisks indicate significant differences.

Previous studies have proved that MR is necessary for stress-induced late-LTP in the DG *in vivo* (Korz and Frey, 2003, 2005b; Ahmed et al., 2006). Nevertheless, these studies only investigated PSA-LTP, but not fEPSP-LTP. The present study tried to verify the essential role of MR also in emotional reinforcement of fEPSP-LTP by i.c.v. infusion of MR antagonist eplerenone 10 min before tetanus/EPF-stress (fig 14 A and B). The results revealed that the blockade of MR activity immediately and severely impaired not only PSA-LTP ($p < 0.001$) but also fEPSP-LTP ($p < 0.001$) in comparison to vehicle group. Remarkably, this impairment was also found in comparison of ERβ-blockade group ($p < 0.001$, each).

4.3.2 The respective application of ERβ and MR antagonists did not have significant effects on gene expression of steroid hormone receptors after EPF-stress

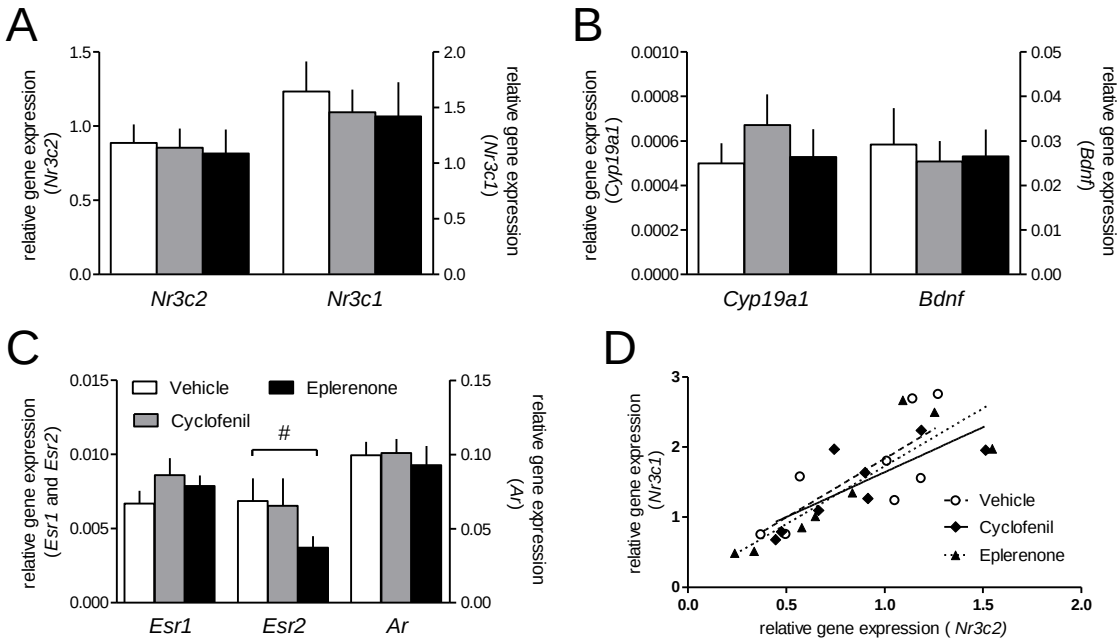

Figure 15. The application of ERβ and MR antagonists did not alter gene expression of steroid hormone receptors 1 h after tetanus/EPF-stress. (A, B, C) No significant difference between groups was detected. Only the expression of ERβ gene *Esr2* in the rats treated with MR antagonist (eplerenone) showed a tendency to be lower than *Esr2* expression in vehicle-treated animals. (D) In all three groups, MR and GR gene expression presented linear correlation without differences in slopes and intercepts. Given are the means and SEM. The number sign indicates the tendency.

According to findings in LTP, we tried to reveal the effects of ERβ- and MR-blockade on hippocampal gene expression of steroid hormone receptors in response to EPF-stress (fig. 15 A, B, C). However, no significant difference was found in gene expression between the three

groups (each group: n = 8). Only a tendency appeared in ERβ expression in the group with MR-antagonist (eplerenone) treatment in comparison to vehicle controls (p = 0.083) (fig. 15 C).

Since a stress-induced linear correlation between MR and GR gene expression was found before (fig. 15 D), linear regression analysis was used to analyze the correlation between MR and GR gene expression in this pharmacological tests with antagonists. Then, linear correlation was found in all groups (vehicle: p = 0.028; cyclofenil: p = 0.018; eplerenone: p = 0.004). But these three lines had no difference in their slopes (p = 0.801) and intercepts (p = 0.802).

4.4 The effects of DNMT and HDAC inhibition

Since the above-mentioned long-lasting repressive effect of MS on MR and ERβ expression as well as LTP-reinforcement, we speculated that DNA methylation may be involved. In order to verify this hypothesis, we applied the DNMT inhibitor 5-aza and HDAC inhibitor TSA via i.c.v. infusion.

4.4.1 DNMT inhibition had no unspecific effects on LTP-reinforcement in MS- rats

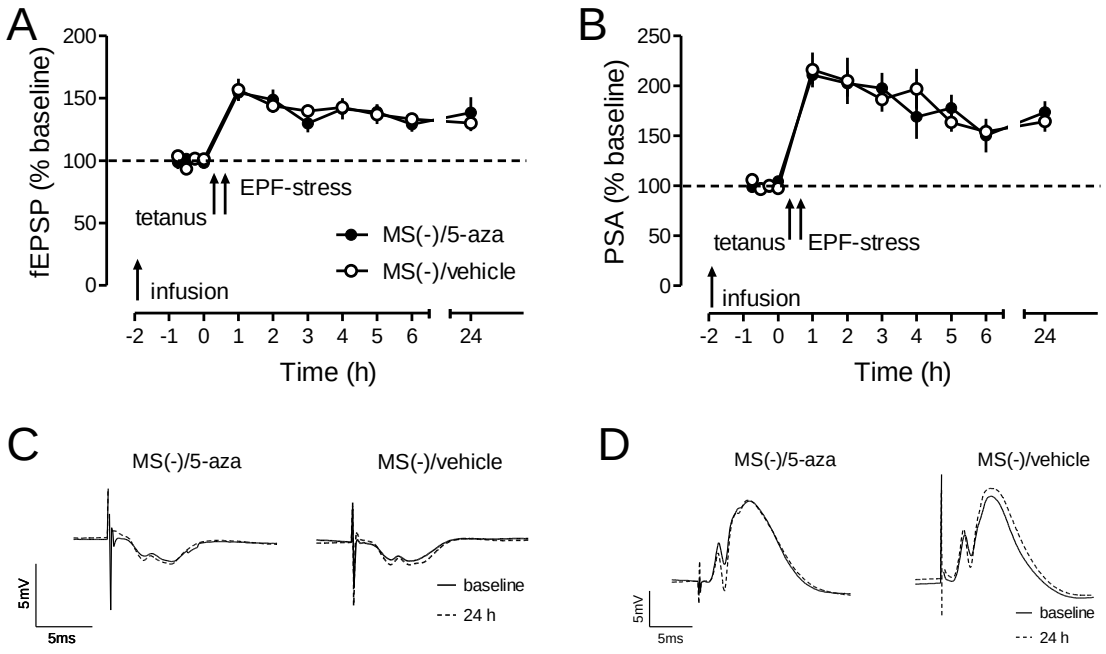

Figure 16. Application of the DNMT inhibitor 5-aza-2'-deoxycytidine (5-aza) did not alter the time course of emotional LTP-reinforcement in control (MS-) rats. (A) The time courses of fEPSP-LTP (5-aza: n = 8, vehicle: n = 8). (B) The time courses of PSA-LTP (5-aza: n = 8, vehicle: n = 8). Representative analog traces of fEPSP and PSA of drug and vehicle treated MS- rats are given in (C) and (D). Given are the means and SEM.

In order to detect possible unspecific shifts in LTP, we first injected 5-aza in MS- rats (n = 8, each). Then, we found no alterations in the time course of LTP of 5-aza treated as compared to vehicle treated rats after post-tetanic stress in both PSA (p = 0.535) and fEPSP (p = 0.310) (fig. 16 A and B).

4.4.2 DNMT inhibition partly restored emotional LTP-reinforcement and gene expression upregulation of ERβ in the hippocampus of MS+ rats

In contrast, the application of 5-aza partly restored the LTP reinforcement in MS+ rats (fig. 17). Although there was no significant difference over the entire time courses of LTP between rats treated with 5-aza and vehicle controls (fEPSP: p = 0.211; PSA: p = 0.129), the PSA curve (fig. 17 A) of the drug treated rats represented a significantly different (reinforced) late phase of LTP (from time point 5 hours after tetanus onwards) compared to the vehicle controls (p = 0.002). In addition, the drug treated rats showed significantly higher fEPSP slopes (fig. 17 B) 24 h after tetanus than the vehicle controls (p = 0.003). Thus, late-LTP was in a certain extent restored by DNMT inhibition in the stressed MS+ rats.

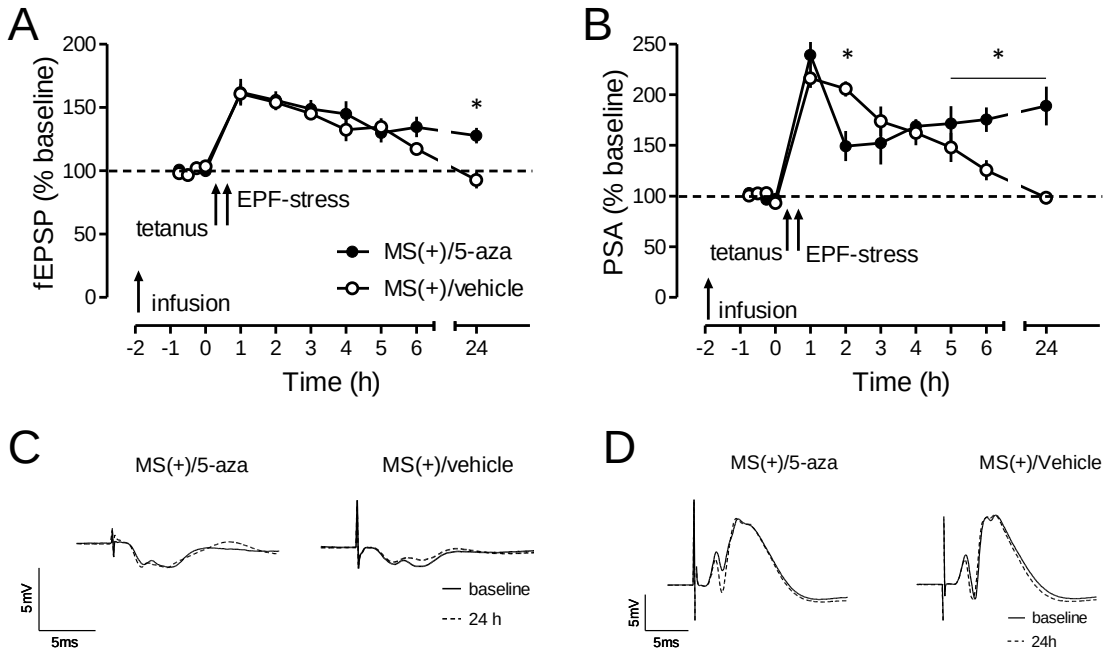

Figure 17. Application of the DNMT inhibitor 5-aza-2'-deoxycytidine (5-aza) 2 h before tetanus/EPF-stress restored LTP-reinforcement in MS+ rats. (A) fEPSP-LTP was significantly reinforced (24 h time point) in MS+ rats treated with 5-aza (n = 8) compared to vehicle controls (n = 6). (B) PSA-LTP was significantly reinforced (5–24 h) in MS+ rats treated with 5-aza (n = 7) as compared to vehicle controls (n = 7). Representative analog traces of fEPSP and PSA of drug and vehicle treated MS+ rats are given in (C) and (D). Given are the means and SEM. Asterisks indicate significant differences.

Corresponding to the rescued LTP-reinforcement, the upregulation of ERβ mRNA was also retrieved by the 5-aza infusion (each group: n = 8; p = 0.040), whereas the hippocampal mRNA level of MR (p = 0.154), GR (p = 0.316), ERα (p = 0.187), AR (p = 0.405), aromatase (p = 0.783) and BDNF (p = 0.117) genes (each group: n = 8) did not show any difference between treated animals and vehicle controls (fig. 18).

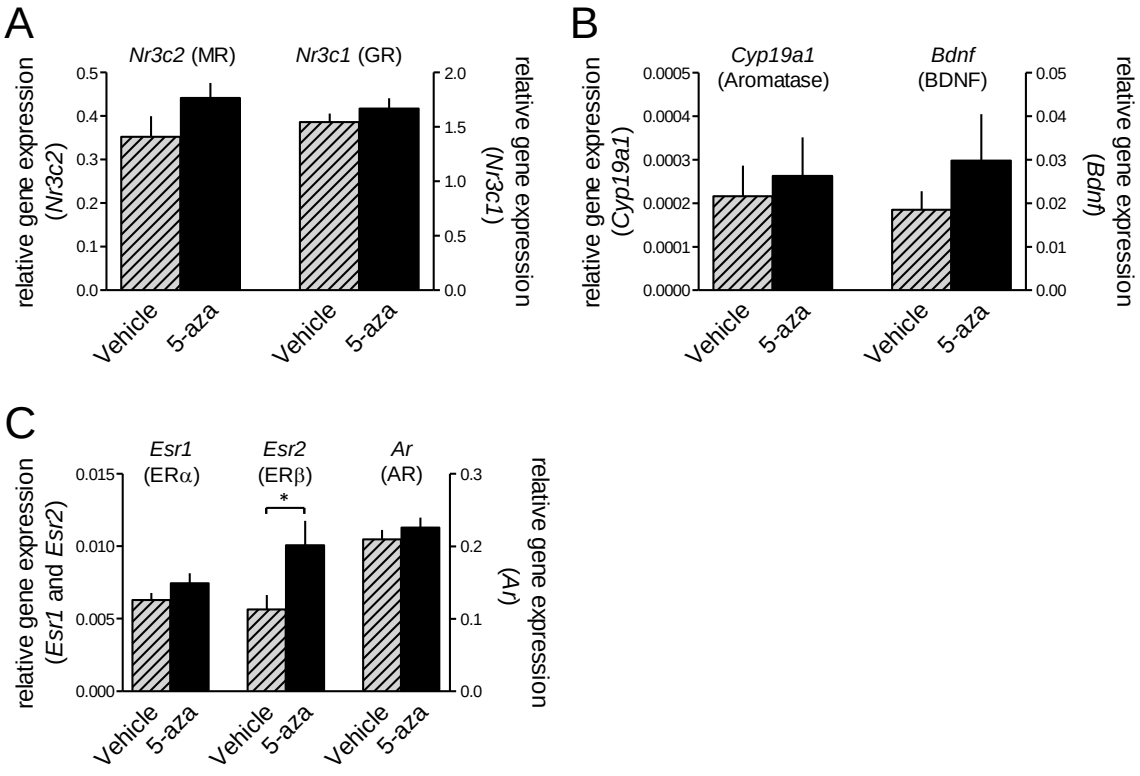

Figure 18. Application of the DNMT inhibitor 5-aza-2'-deoxycytidine (5-aza) via i.c.v. infusion 2 h before tetanus/EPF-stress restored the upregulation of ERβ gene expression. (A) Infusion of 5-aza did not restore the stress-induced hippocampal upregulation of MR expression in MS+ rats. (B) No effects on aromatase and BDNF gene expression were detected. (C) However, 5-aza application retrieved the upregulation of hippocampal ERβ gene expression 1 h after tetanus/EPF-stress in MS+ rats (n = 8, for each group and each gene). Given are the means and SEM. Asterisks indicate significant differences.

4.4.3 The effects of HDAC inhibition

In contrast to DNMT inhibition, the i.c.v. infusion of the HDAC inhibitor TSA 2 h before tetanus/stress (fig. 19) did not counteract the effect of MS and did not reinstate the emotional LTP reinforcement in MS+ rats both for PSA (p = 0.535) and fEPSP (P = 0.310).

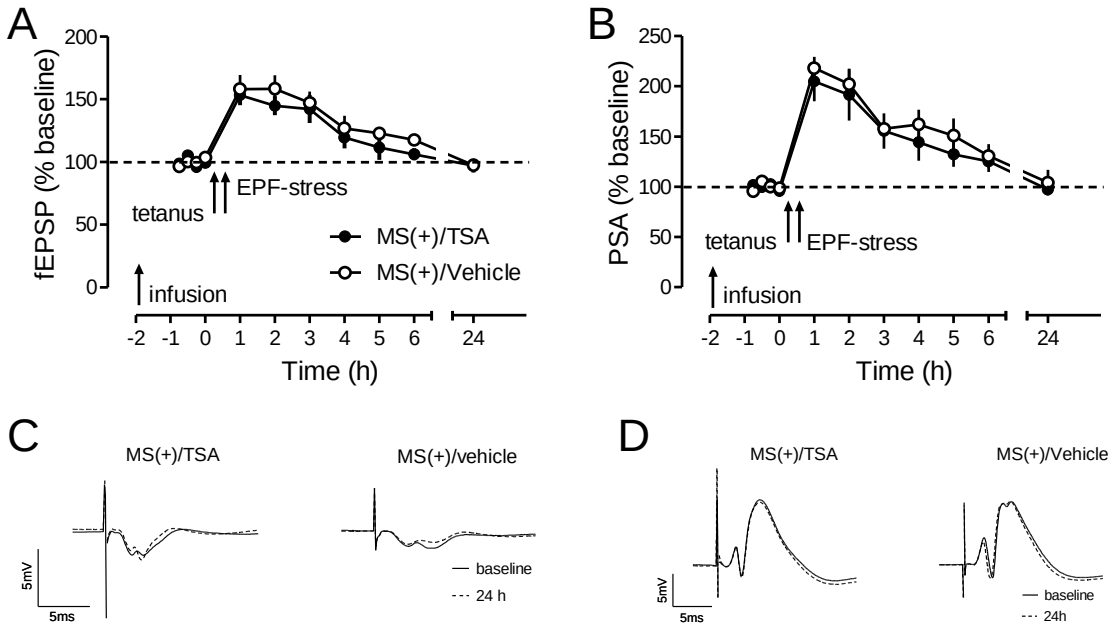

Figure 19. Application of the HDAC inhibitor trichostatin A (TSA) 2 h before tetanus and EPF-stress did not restore the impaired EPF-stress induced LTP-reinforcement in MS+ rats. MS+ rats treated with TSA and vehicle controls showed the same time course of both fEPSP- (A, TSA: n = 8, vehicle: n = 7) and PSA-LTP (B, TSA: n = 8, vehicle: n = 7) after EPF-stress. Representative analog traces of the fEPSP and the PSA of drug and vehicle treated MS+ rats are depicted in (C) and (D). Given are the means and SEM.

4.5 MS modified DNA methylation level in Esr2 promoter region

The effects of DNMT inhibition on ERβ gene expression in MS+ rats suggest the involvement of DNA methylation in the long-lasting or acutely induced effects of our MS paradigm. Therefore, DNA methylation assays for *Esr2* promoter region were performed on unstressed and stressed MS+ and MS- rats (each group: n = 10). CpG site 20 showed an upregulated methylation level, while the methylation level of position 30 was downregulated in methylation levels in MS+ as compared to control rats (fig. 20 A). The general methylation level (mean value of average methylation percentage for all CpG sites) showed no differences (fig. 20 C). These results indicated no significant changes induced by MS under basal conditions.

Nevertheless, MS slightly, but significantly, lowered general methylation level in *Esr2* promoter region after EPF-stress (fig. 20 C). For single CpG sites, 8 positions (position 3, 12, 13, 14, 16, 17, 18, 26) showed a lower methylation level in MS+ than MS- animals, whereas only position 6 had higher methylation level in MS+ group (fig. 20 B). Among the positions

with difference, 6 CpG sites (position 12, 13, 14, 16, 17, 18) were localized in the region from -320 to -179 in reference to transcription start site (TSS) (fig. 6). These results verified that MS can induce some kinds of changes in DNA methylation of *Esr2* promoter after acute stress conditions in adulthood.

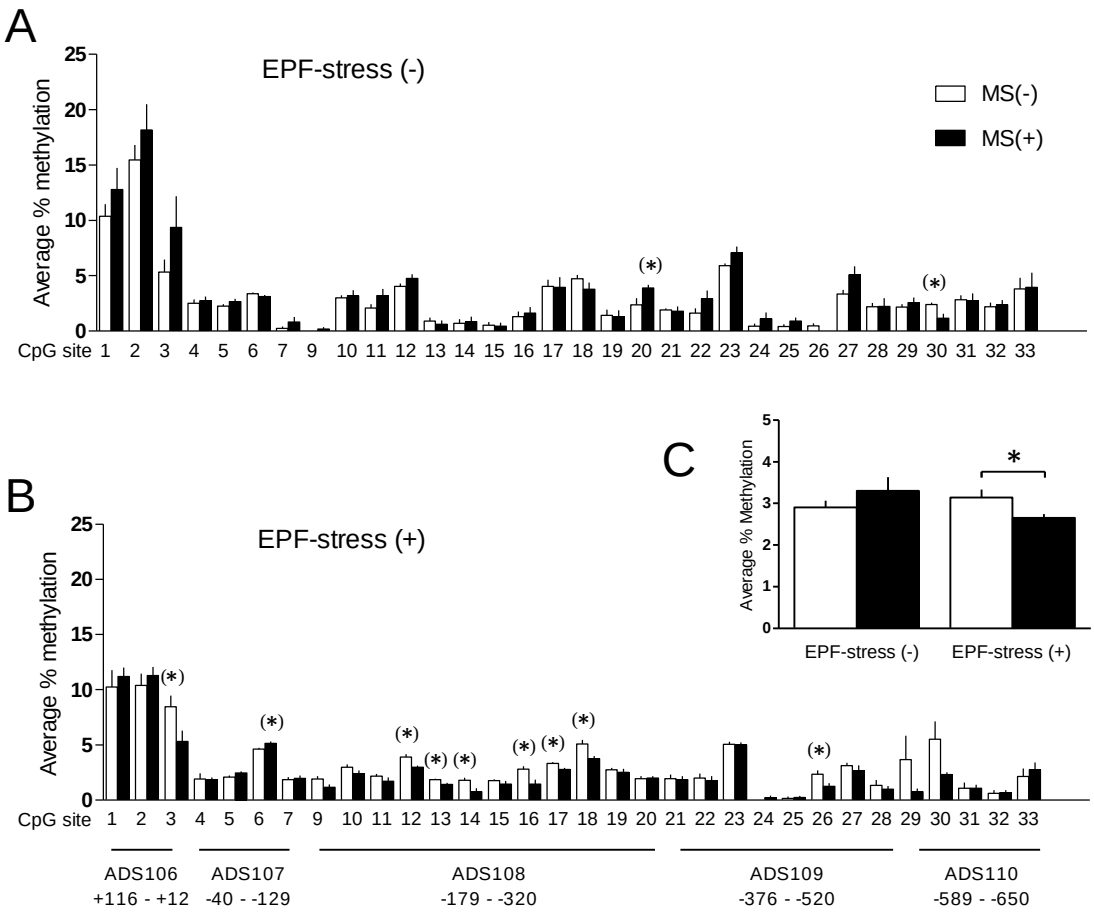

Figure 20. Maternal separation modified DNA methylation level in ERβ gene (*Esr2*) promoter region in the hippocampus. (A) Under basal conditions (without EPF-stress), MS+ rats (n = 10) showed only two CpG sites with differences compared to MS- rats (n = 10). One had a higher level and the other showed a lower level of methylation. (B) After EPF-stress, 8 CpG sites showed higher methylation level in MS+ than MS- group, while position 6 had lower methylation level in MS+ (n = 10) than MS- rats (n = 10). All significances (A, B) were analyzed without Bonferroni correction. After Bonferroni correction, there were no differences in single sites. (C) The general methylation level in MS+ group was also significant lower than MS- group after adult stress. There were no significant differences in general methylation levels between the other groups. DNA methylation levels were presented as average percent methylation. Given are the means and SEM. Asterisks with brackets indicate significant differences without Bonferroni correction. Due to technical malfunction, the data of CpG site 8 were not reliable and not presented in the graphics.

4.6 The effects of MS on behavior in adulthood

Previous studies prove that early-life stress alters also behavior, learning and memory formation. The above-mentioned experiments revealed the effects of MS on hippocampal plasticity and gene expression. Then, we wanted to verify whether these neurobiological changes can affect the behavioral output of early-stressed rats. Previous studies indicate a critical role of steroid hormones and their receptors in depression (McEwen, 2010, 2011), while our results have shown an altered MR and ERβ expression in response to adult stress in early-stressed animals. Therefore, a difference in anxiety-like behavior between groups was expected and elevated plus-maze (EPM) test was applied.

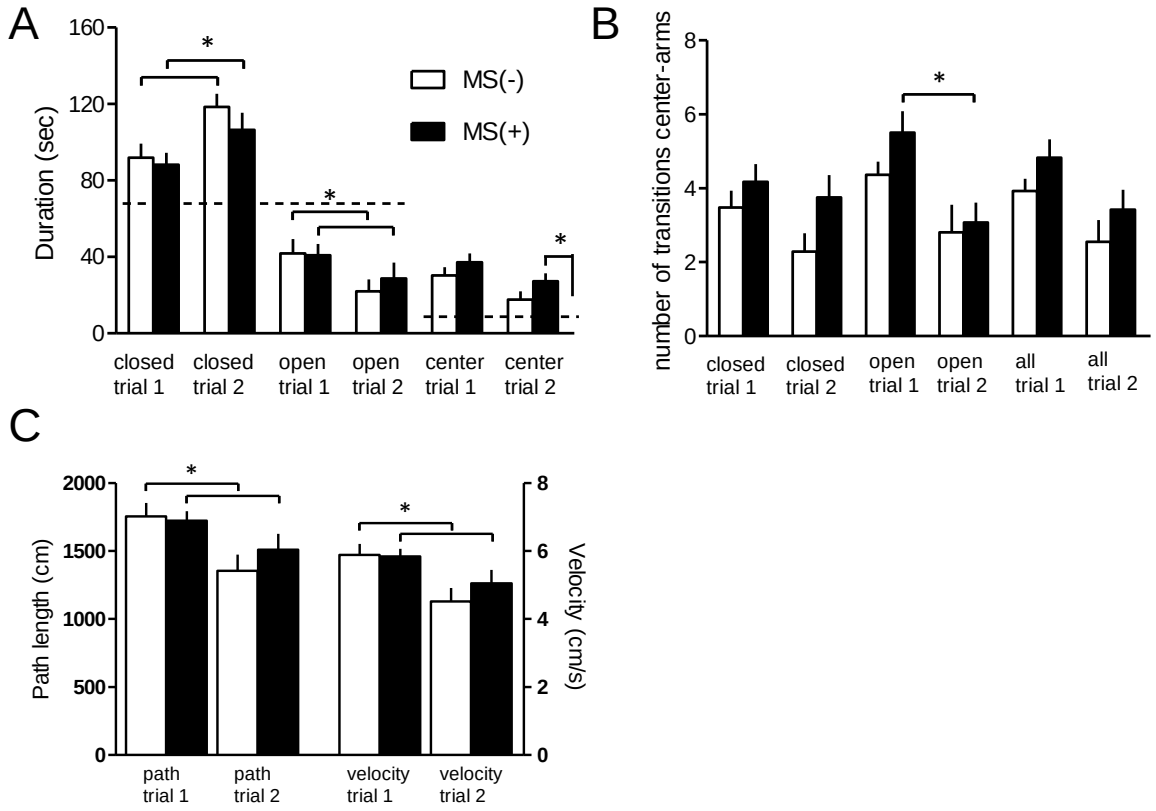

Figure 21. MS+ rats showed impaired anxiety-like behavior in elevated plus-maze (EPM) test than MS- rats. (A) Both groups of rats took longer time in the closed arms and less time in the open arms in trial 2 than trial 1. The time that MS+ (n = 18) rats took in the center of EPM in the second trial was significantly beyond the random distribution in the center (dashed line, 14.1±1 s). (B) In trial 2, MS+ rats showed a lower transition-number from center to open arms, whereas MS- rats did not show this behavior. (C) In trial 2, both MS+ and MS- rats (n = 21) presented lower path length and velocity than trial 1. Given are the means and SEM. Asterisks indicate significant differences.

4.6.1 MS+ rats showed a potential lower anxiety than MS- rats

No significant differences in main behavioral parameters were found between MS+ and MS- rats. Interestingly, both groups of animals appeared to be more anxious in the second trial (fig. 21 A) according to the increased duration in the closed arms (MS+: $p = 0.011$; MS-: $p = 0.001$) and decreased duration in the open arms (MS+: $p = 0.004$; MS-: $p = 0.002$), whereas the durations in the center region were not different between trial 1 and trial 2 (MS+: $p = 0.163$; MS-: $n = 0.178$). But there was no difference between MS+ and MS- rats (for each comparison $p > 0.01$) in the duration in every subregion of the EPM. However, MS+ rats appeared to have a higher motivation to try the open arm in trial 2, because the time they spent in the center of the maze was significantly beyond the random distribution in the center region (14.1 s) in trial 2 ($p = 0.045$). But the duration of MS- rats in the center is not beyond the random distribution ($p = 0.875$) (fig. 21 A). The higher motivation of MS+ rats to stay in the center, which is also an open field, implicates a potential lower level of anxiety in comparison to MS- rats. In addition, MS+ rats showed a lower transition-number from center to open arms in trial 2 contrasted to trial 1 ($p = 0.035$), while no difference (but a tendency) in transition-number from center to open arms between two trials was found in MS- rats ($p = 0.056$) (fig. 21 B). With regard to locomotion, both groups of rats showed reduced path length (MS+: $p = 0.011$; MS-: $p < 0.001$) and velocity (MS+: $p < 0.001$; MS-: $p = 0.001$) during trial 2 (fig. 21 C). There were no significant differences between MS+ and MS- rats in path length and velocity in both trials (for each comparison $p > 0.013$).

4.6.2 MS+ rats showed lower corticosterone level in serum after EPM

Interestingly, these detected differences in behavior may be attributed to the altered HPA axis activity by MS, since hormone analyses then identified that MS+ rats had lower level of serum corticosterone than MS- animals 15 min after the second trial of EPM ($p < 0.001$), while no difference between groups was found in the basal level ($p = 0.763$; $n = 8$ each) (fig. 22 A). However, serum corticosterone level 15 min after EPF-stress ($n = 8$ each) was not different between groups ($p = 0.750$). This observation may implicate that our MS+ rats show the same responsiveness to acute stress, but a quicker habituation to stressful events in adulthood. The hippocampal corticosterone level after EPM (fig. 22 B) was not significantly different between groups (MS+: $n = 8$; MS-: $n = 11$; $p = 0.305$).

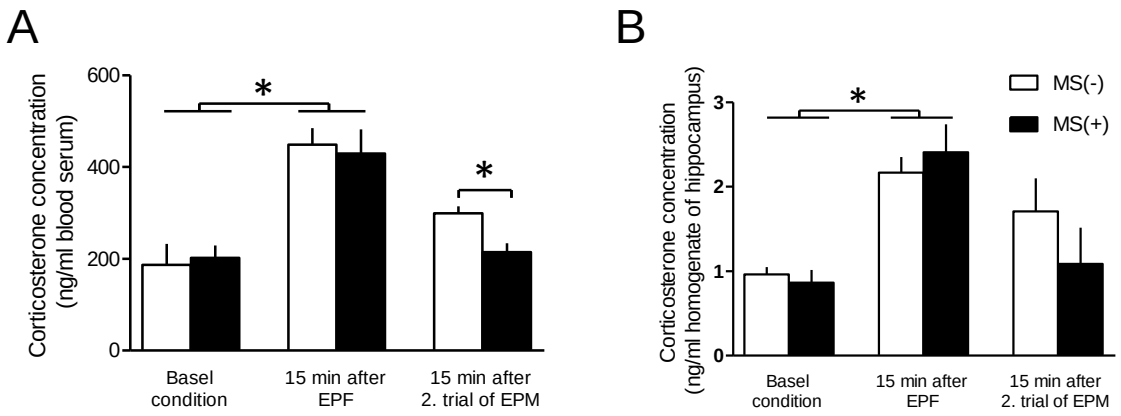

Figure 22. MS- rats had lower serum corticosterone level than MS- rats after EPM test. (A) Fifteen min after the second trial of EPM, MS+ rats (n = 18) showed a significant lower level of serum corticosterone than MS- rats (n = 21), while this difference were not detected under non-stressed condition and after EPF-stress. (B) Corticosterone level in the hippocampus was not significantly different between MS+ (n = 8) and MS- rats (n = 11) after EPM test. Given are the means and SEM. Asterisks indicate significant differences.

5. Discussion

The present study aimed to reveal the effects of early-life stress on hippocampal LTP in young adults and the underlying mechanisms. Then, we found that repeated MS from PND14 to 16 for everyday 6 h had no effect on LTP induced exclusively by high-frequency stimulation (HFS). However, this MS paradigm prevented LTP-reinforcement induced by post-tetanic stress in adulthood. Further experiments proved that MS also impaired the upregulation of MR and ERβ gene expression in response to post-tetanic stress. This result implicates the MR and ERβ as critical factors for the formation and maintenance of stress-induced late-LTP, which was then proved to be protein-synthesis-dependent. The pharmacological treatment with the antagonists of MR and ERβ then verified their critical roles in emotional LTP-reinforcement. In order to investigate the involvement of epigenetic mechanisms in the effects of MS, DNMT and HDAC inhibitors were applied. The inhibition of DNMT restored both emotional LTP-reinforcement and adult-stress-induced upregulation of ERβ gene expression. The further DNA methylation analysis verified the MS-induced changes of DNA methylation pattern within the *Esr2* promoter region (Wang et al., 2012). These findings in neurophysiology, gene expression and epigenetic modifications were in a certain extent supported by the results gained in behavioral tests with EPM. The early-stressed animals appeared to have a lower risk of anxiety than controls, although their differences in behavior throughout EPM tests were only slight. Based on these results, the following inferences are drawn:

1. MS from PND14 to 16 for everyday 6 h in rats can reduce the physiological sensitivity to emotional stimulation in the hippocampus during adulthood.

2. Receptors of both corticosteroids and gonadal steroids are involved in the regulation of hippocampal synaptic plasticity in response to emotional stimulations.

3. MS can carry out its long-lasting effects on hippocampal plasticity by changing DNA methylation level in response to acute stress in allusion to certain genes in the hippocampus.

4. The above-mentioned effects of MS may be associated to a decreased risk of anxiety in adulthood.

In the following part of discussion, these results and inferences will be interpreted in detail and the significance of our research for future investigations will be elucidated.

5.1 The effects of MS on hippocampal LTP

Previous studies have revealed that MS can permanently alter the activity of HPA axis and hippocampal plasticity (Meaney et al., 1996; Lehmann et al., 1999; Aisa et al., 2007). Recent results also indicate that low level of maternal care and maternal deprivation can impair stress-induced LTP modification in adulthood (Champagne et al., 2008; Bagot et al., 2009; Oomen et al., 2010). The present study also revealed that MS from PND 14 to 16 for everyday 6 hours leads to a suppressed LTP-reinforcement induced by post-tetanic stress in adulthood. In contrast, our MS paradigm did not affect the late-LTP induced by electrical strong tetanus, suggesting that MS did not disturb the mechanisms for late-LTP formation induced by acute stress, but the stress-responsiveness of the hippocampus. Our results are consistent with the previous findings that low level (or loss) of maternal care can lead to lower responsiveness of the hippocampus to stressors in the offspring later in life (Champagne et al., 2008; Bagot et al., 2009; Oomen et al., 2010).

Since DNMT inhibition was found to enhance the suppressed LTP-reinforcement in MS+ rats, MS likely achieved its impairment effect on LTP in adulthood by modulating DNA methylation pattern of the genes related to late-LTP formation. Previously, DNMT inhibition by 5-aza has been shown to impair hippocampal early-LTP *in vitro* within the CA1 region (Miller et al. 2008). This finding seems to be contradictory to our results. This may be due to the fact that DG-LTP partly involves different mechanisms than CA1-LTP. These two substructures of the hippocampus receive different heterosynaptic inputs. For example, dopamine-mediated glycoprotein-processing is necessary for late-LTP formation in the CA1 neurons (Angenstein et al., 1992), whereas DG-LTP more depends on norepinephrine signalling (Stanton and Sarvey, 1985), but not dopaminergic heterosynaptic receptor activation (Ahmed et al., 2006). The transient effect on early-LTP in MS+ rats may be related to different pre-methylation states in these animals. MS has been reported to induce hypermethylation states in adulthood throughout the hippocampus (Mychasiuk et al. 2011). Although the DNA methylation assays in the present study did not reveal any difference between MS+ and MS- rats in ERβ gene promoter region, the basic methylation status of the MS+ rats in our studies is still very likely different from MS- rats in many other regions of the genome, some of which may play critical roles in the regulation of synaptic plasticity.

5.2 The effects of MS on HPA axis

The above-mentioned effects of MS on LTP implicate that our MS paradigm may alter HPA activity and hippocampal response to stress in adulthood. Hormone and gene expression

analyses then verified this inference. A variety of previously used paradigms of early aversive experiences result in alterations of hippocampal MR/GR activities even under basal conditions in adult animals (Vazquez et al. 1996; Weaver et al. 2001). In contrast, we found a specific repression of the MR gene expression only after stress in adulthood, whereas GR expression and the hippocampal corticosterone content were not significantly different between MS+ and MS- rats. These results indicate once again that MS can have a long-term effect on HPA axis activity, whereas MS in different time windows may alter HPA activity and stress-responsiveness differently. Since DNMT inhibition did not restore MR gene expression in MS+ rats, the mechanisms underlying the MS-induced change of hippocampal MR expression are still not clear.

Interestingly, although EPF-stress did not induce differences in corticosterone level between MS+ and MS- rats, the serum corticosterone level after the second trial of EPM test was lower in MS+ than MS- animals. This result reveals a quicker habituation to stressful events of early-stressed rats. Since gene expression analysis was not performed in the animals with EPM tests, more investigations are required for revealing the detailed mechanisms of the difference in corticosterone release. But according to the effects of MS on hippocampal ERβ gene expression, ERβ and the paraventricular nucleus (PVN) of the hypothalamus, which plays a key role in controlling HPA activity and expresses a high level of ERβ (Shughrue et al., 1997; Alves et al., 1998; Greco et al., 2001), is likely involved in the MS-induced alterations in stress-habituation.

5.3 The effects of MS on HPG axis and the role of DNA methylation in these effects

In contrast to stress hormones and their receptors, the effects of early-life stress on the release (or synthesis) of sex hormones and the gene expression of their receptors are poorly revealed by previous studies. But the sex differences in response to MS (Viveros et al., 2009; Llorente et al., 2011) imply the involvement of the interaction between HPA- and HPG-axis in the effects of MS. The present study revealed direct evidences that MS can affect the gene expression level of ERβ in the hippocampus in adulthood and this alteration of gene expression is related to DNA methylation modifications. Our MS paradigm repressed the upregulation of *Esr2* expression after adult stress (EPF-stress) and DNMT inhibition reverted this upregulation in MS+ rats, suggesting that MS achieved its regulation on *Esr2* expression by increasing DNA methylation level. However, the DNA methylation assays surprisingly found no general higher methylation level in *Esr2* promoter region in MS+ rats as compared to MS- rats, but a mild downregulation of the mean methylation level. This result on the first

hand proves that our MS-paradigm can induce modifications in DNA methylation patterns in response to stress in adulthood, but it seem to be not content with the finding in our previous gene expression analysis.

Nevertheless, the application of DNMT inhibitor can generally reduce the activity of DNMT and methylation level in the whole genome. Many genes could be involved. Therefore, the restored upregulation of *Esr2* expression in MS+ rats may be due to the upregulation of the transcription factors promoting *Esr2* expression. Similarly, the stress-induced upregulation of ERβ gene expression in MS- rats may also be regulated by the same transcription factors.

On the other hand, different transcription factors can interact with respect to the activation or repression of transcription, which may depend on their different conformations and specific binding sequences in the genome. A number of sequence-dependent transcriptional repressors and their specific binding sequences within promoter regions of certain genes have been identified, such as Heat shock factor 1 (Singh et al., 2002), Snail (Battle et al., 2000) and BCL-6 (Chang et al., 1996). Different with the methyl-binding proteins, such as methyl CpG binding protein 2 (MeCP2) and methyl-CpG-binding domain proteins (MBD), which bind with methylated CpG islands without the recognition of specific sequences (Hendrich and Bird, 1998; Chahrour et al., 2008), sequence-dependent transcription repressors have their specific binding sequences in the genome, usually within the promoter regions, such as enhancer boxes, to prevent gene transcription. Whether the increased DNA methylation can block the recognition of these transcriptional repressors to their specific binding regions and enhance gene expression is still unknown. Accordingly, these findings reveal that transcription can be effectively fine tuned in response to specific acute tasks or challenges and that is possible even by small changes in methylation levels (Jaenisch and Bird, 2003).

In addition, more and more evidences demonstrate DNA as allosteric ligands for nuclear receptors (Hall et al., 2002; Meijsing et al., 2009). It means that the same nuclear receptor acting as transcription factor may play different roles in regulation of gene expression, when it binds to different DNA sequences, since the binding with different sequences may lead to different conformations of this transcription factor and then alter its function (positive or negative for gene transcription). This modulation of conformation and function has been found in GR (Meijsing et al., 2009) and ERβ (Heery et al., 1997; Hall et al., 2002). All these findings indicate that transcription factors not only enhance but also repress gene expression, depending on their conformations and binding sequences. Thus, the effects of DNA methylation on gene transcription are likely not “monophonic” but “stereo”. Although adult

stress induced a decreased methylation of *Esr2* in our study, this decrease may not be opposite to repressed gene expression.

With regard to our results, the most CpG sites with different methylation level between MS- and MS+ rats are localized in cluster ADS108 from -329 to -118 in relation to the TSS (fig. 6), suggesting this region as a critical binding site for potential transcription regulators. Whether this region is the specific binding sequence for a transcriptional repressor requires further researches to prove. At present, it cannot be decided, whether the MS-induced regulation on *Esr2* expression is due to the methylation changes or to the regulation on the genes of specific transcription factors binding to *Esr2* promoter.

5.4 The role of corticosteroid receptors in the emotional LTP-reinforcement

5.4.1 MR activity regulates both input and output activity of hippocampal neurons after stress

Previous studies have proved that MR activation in a certain time-window around LTP-induction is necessary for emotional LTP-reinforcement *in vivo* (Korz and Frey, 2003, 2005b). In these studies, only population spike was recorded and the i.c.v. infusion of an MR antagonist was found to severely impair PSA-LTP (Korz and Frey, 2003, 2005b). The present study verified that not only the reinforcement of PSA- but also fEPSP-LTP was dependent on MR activity, when acute stress takes place immediately after tetanus. Moreover, the MR-dependence of fEPSP-LTP seems to be more intensive, because failed upregulation of MR expression leaded to a weak reinforcement of fEPSP-LTP and only MR antagonist (but not ERβ antagonist) was effective on fEPSP-LTP. Thus, MR seems to very effectively control input and output activity shortly after stress, whereas later phases are controlled in a more complex manner.

However, our results that DNMT-inhibition was effective in the restoration of late-LTP, but not the upregulation of MR-mRNA after stress (although there is a slight non-significant increase in the DNMT-inhibitor treated group), seem to be contrary to the previous evidences for the MR-dependence of stress-induced late-LTP. There is at least one argument to negate this question: previously, MRs were inactivated by receptor antagonists, which result in a saturation of receptors and an immediate depotentiation of the PSA-LTP (Korz and Frey, 2003; Schulz and Korz, 2010b). But in the present study, although the expression of MR was reduced in MS+ rats, there was still MR activity, which is still able to at least partly regulate down-stream factors for late-LTP formation. So our results do not contradict the previous studies. But we cannot judge that the upregulation of MR gene expression does not play a role.

Actually, the temporary depotentiation of PSA-LTP 2 h after tetanus/EPF-stress under 5-aza in only early-life-stressed animals may be attributed to deficient MR expression. The fEPSP-LTP was less affected by MR blockade in an earlier study with a stress exposition 15 min post-tetanus (Schulz and Korz, 2010b).

5.4.2 Extranuclear MR may play the main role in emotional LTP-reinforcement

In the present study, it was revealed that the blockade of MR activity suppresses not only late-LTP, but also early-LTP. This immediate depotentiation of LTP is reported in almost all *in vivo* LTP-studies applying MR antagonists (Korz and Frey, 2003, 2005b; Ahmed et al., 2006; Avital et al., 2006; Schulz and Korz, 2010b). However, only late-LTP depends on gene expression and protein synthesis (Krug et al., 1984; Frey et al., 1988; Reymann et al., 1988a; Otani and Abraham, 1989; Otani et al., 1989; Manahan-Vaughan et al., 2000). Since steroid hormones are able to pass through plasma membranes, the classical working mode of MR is described as direct regulation of gene expression in cell nuclei. This action takes hours to days to manifest (McEwen and Alves, 1999; Brinton, 2009). So theoretically, MR blockade should only affect late-LTP. Therefore, the rapid effect of MR blockade on LTP suggests the involvement of extranuclear MR in LTP induction, whereas the effects on late-LTP are mainly attributed to the genomic activity of MR.

Growing evidences have verified the rapid corticosteroid signalling in the brain (Hinz and Hirschelmann et al., 2000; Evanson et al., 2010) and the non-genomic actions of MR (Atkinson et al., 2008) and GR (Solito et al., 2003). A recent study has reported that the introduction of corticosterone into hippocampal CA1 pyramidal cells can increase the frequency of miniature excitatory postsynaptic current (mEPSC) and this rapid effect of corticosterone dispears in the *Nr3c2*-knockout mice (Karst et al., 2005). These findings are suggestive of the dependence of rapid stress-response in the hippocampus on the extranuclear cortisol signalling (Hammes and Levin, 2007). Furthermore, it has been shown that the blockade of MR had no effect on fEPSP-LTP, when stress exposition takes place 15 min after LTP induction (Schulz and Korz, 2010b). Thus, MR seems to achieve non-genomic mechanisms inducing intracellular signalling cascades that then regulate late-LTP by the induction of more complex gene expression patterns and/or protein synthesis processes. Therefore, non-genomic MR may function as a molecular switch controlling the input and output neuronal activity time dependently during acute stress events, whereas ERβ exerts more genomic functions due to the regulation of late-LTP.

In addition, MR blockade not only showed a rapid effect on early-LTP, but also a long-lasting effect on late-LTP, which is protein-synthesis-dependent. It is suggestive of a genomic role of MR in LTP-reinforcement. This role may be accomplished by classical MR, directly enhancing gene transcription, or by activating other transcription factors. Aldosterone-induced activation of MAPK pathway is proved to depend on MR (Olijslagers et al., 2008). Therefore, MR blockade can also reduce the activity of CREB, which is a downstream factor in MAPK pathway.

5.4.3 The interaction between MR and GR in the regulation of synaptic plasticity

Previous results revealed an opposite role of GR in stress-induced modifications of hippocampal synaptic plasticity, in contrast to MR (Pavlides et al. 1995, 1996; Kim and Yoon 1998). However a critical role of the relative MR/GR occupation ratio is more likely with respect to neuronal and synaptic responsiveness to stress, spatial learning and memory (de Kloet et al. 1998; Bilang-Bleuel et al. 2005; Calvo et al. 2011). Our results support this view and further indicate that the lopsided expression of MR and GR in the hippocampus may be one reason for the failed LTP-reinforcement in response to stress.

According to our analysis on the correlation between hippocampal MR and GR gene expression, there is no correlation between these two genes without stress, whereas MR/GR expression presents a linear correlation after EPF-stress in both MS- and MS+ animals. But the intercept of the line for MS+ group (showing no late-LTP) is significantly lower than that for control rats (showing late-LTP). The balance betweenMR and GR plays a crucial role in the regulation of stress responsiveness, while a disturbance of this balance can induce a dysfunction of the stress-responsive system and a vulnerability to stress-related disorders (de Kloet et al., 2005). Thus, the impaired LTP-reinforcement in our study is also related to the unbalanced MR/GR expression, supporting the view that MR and GR work in concert to regulate hippocampal synaptic plasticity in response to stress.

5.5The role of ERβ in the emotional LTP-reinforcement

The most remarkable finding of the present study is that acute stress can induce the upregulation of ERβ expression in the hippocampus of male rats, while the application of specific antagonists proved that the activity of ERβ plays a crucial role in the emotional reinforcement of LTP, but only in PSA-LTP. These results are in line with the findings in *Esr2*-knockout mice, which showed an attenuated LTP in the CA1 region of the hippocampus (Day et al., 2005). The exclusive effect of ERβ blockade on PSA-LTP coincides with the

stronger effect of retrieved *Esr2* upregulation with the failed upregulation of MR gene on PSA- than fEPSP-LTP after DNMT inhibition. In contrast, MR is necessary for the reinforcement of both PSA- and fEPSP-LTP. Previous studies have reported the role of MR in emotional LTP-reinforcement (Korz and Frey, 2003, 2005b). Our results verified that sex hormone receptors like ERβ can also play an important role in stress-induced late-LTP in the hippocampus.

5.5.1 ERβ can regulate-LTP by transcriptional and translational ways

In the present study, the effects of the ERβ antagonist cyclofenil appeared significantly later than that of the MR-antagonist, though both drugs were infused 10 min before tetanus. The blockade of ERβ only suppressed late-LTP, which depends on protein synthesis. The specific role of ERβ in late-LTP formation suggests the involvement of protein synthesis and/or gene expression in the regulatory effects of ERβ on late-LTP formation, whereas MR may function more through the ways of post-translational modification.

The classical role of ERs is their genomic effects on gene expression regulation. For example, acute and chronic estrogen treatments were found to increase 5-HT_{2A} receptor mRNA in the dorsal raphe nucleus and 5-HT_{2A} receptor binding in various brain regions in ovariectomized female rats (Sumner and Fink, 1993, 1995; Fink and Sumner, 1996; Cyr et al., 1998), whereas these effects could be reversed by the ERβ antagonist tamoxifen, suggesting that these effects of estrogen treatment are mediated by genomic ERβ (Sumner et al., 1999). The activation of 5-HT receptors is necessary for emotional LTP-reinforcement (Ahmed et al., 2006). Accordingly, ERβ antagonists may suppress late-LTP by reducing gene expression of 5-HT receptors, whereas the upregulation of ERβ expression may enhance LTP by increasing 5-HT level in the hippocampus. Previous studies also revealed that ovariectomized rats showed decreased gene expression of the dopamine transporter (DAT) in the substantia nigra pars compacta (SNc) (Bossé et al., 1997), suggesting a regulatory role of genomic ERs in DAT gene expression. The activation of dopamine receptors D1/D5 has been proved to enhance LTP expression in the DG (Kulla and Manahan-Vaughan, 2000). DAT reuptakes extracellular dopamine back to cytosol and plays a critical role in dopamine signalling. Thus, ERβ may affect dopaminergic heterosynaptic inputs and signalling in the hippocampus.

Moreover, electron microscopic studies also identified that ERβ was not only located in cell nuclei, but also affiliated with cytoplasmic organelles, especially endomembranes and mitochondria (Milner et al., 2005). These findings suggest that ERβ may not only be involved

in the regulation of gene expression, but also can direct affect mRNA translation, on which late-LTP is dependent. This inference is supported by the finding that estrogen treatment can rapidly increase the phosphorylation of eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (Akama and McEwen, 2003).

The translation of AMPA subunit GluR1, synaptophysinand postsynaptic density-95 (PSD-95) has been found to be increased after ERβ activation by a selective agonist (Liu et al., 2008; Waters et al., 2009). AMPA is without question an integrant molecule for LTP induction (Herron et al., 1986; Reymann et al., 1988b; Zamanillo et al., 1999; Boehm et al., 2006), while PSD-95 and synaptophysin are also found to play a crucial role in synaptic plasticity (Janz et al., 1999; Beique and Andrade, 2003; Ehrlich et al., 2007). PSD-95 is a member of the membrane-associated guanylate kinase (MAGUK) family. This protein can enhance the phosphorylation of NMDA receptors (Tezuka et al., 1999) and is required by the LTP-induction-dependent spine growth (Steiner et al., 2008). Synaptophysin is a major synaptic vesicle protein, which is upreglated after LTP induction in the DG, suggesting a critical role in LTP induction (Mullany and Lynch, 1997). Accordingly, ERβ may accomplish its regulation of synaptic plasticity by increasing the level of the above-mentioned molecules.

However, the above-mentioned effects of ERβ on the PRPs appear quite quickly after ERβ agonist application (Liu et al., 2008; Waters et al., 2009). In contrast to this, we found that ERβ-antagonists took effects 4 hours after infusion. This observation may be explained by the dependence of stress-induced late-LTP on ERβ gene expression upregulation. The upregulated mRNA of ERβ requires time to be translated into functional proteins. ERβ can regulate synaptic plasticity just when enough ERβ proteins were synthesized. Thus, the infused cyclofenil did not take its effect immediately. The half-life of cyclofenil is 29 h after oral administration to men (Borgström, 1981).

5.5.2 ERβ can regulate-LTP by post-transcriptional modification

Electron microscopic studies have detected ERs affiliated with endoplasmic reticulum (Milner et al., 2001, 2005; Mitterling et al., 2010). This location suggests that ERs can not only affect gene expression and protein synthesis, but also mediate rapid estrogen signalling by post-translational modifications in the CNS (Balthazart and Ball, 2006; Garcia-Segura, 2008; Srivastava et al., 2011). These extranuclear-ER-dependent signalling pathways may also contribute to emotional LTP-reinforcement. Growing evidences have indicated that estrogens can affect cognition and behavior, as well as cellular and molecular events, rapidly (Srivastava et al., 2011).

Previous studies have revealed that both ERα and ERβ are able to activate metabotropic glutamate receptors (mGluR) without ligand (glutamate), leading to down-stream second messenger signalling (Boulware et al., 2005; Grove-Strawser et al., 2010). This interaction between ERs and mGluR has been found in hippocampal neurons (Srivastava et al., 2011). The critical role of mGluR in LTP-induction has been reported. The non selective group I/group II mGluR antagonist (S)-α-Methyl-4-carboxyphenylglycine ((S)-MCPG) can block LTP-induction in the DG *in vivo* (Riedel and Reymann, 1993; Riedel et al., 1995), while the selective mGluR group I agonist trans-azetidine-2,4-dicarboxylic acid (ADA) can reinforce a short-term potentiation induced by weak tetanus into LTP (Manahan-Vaughan and Reymann, 1996). According to these findings, ERβ blockade may impair LTP by the reduction of mGluR activity. Moreover, the activation of mGluR can subsequently activate PKC and CaMKII (Choe and Wang, 2001). Therefore, ERs can also activate PKC and modify newly synthesized transcription factors, such as CREB, through the interaction with mGluR (Boulware et al., 2007). Through these transcription factors, ERβ can accomplish its regulatory effects on gene expression and contribute to late-LTP formation.

In addition, other mechanisms may be also involved in the post-translational functions of extranuclear ERβ in emotional LTP-reinforcement. There is evidence that rapid estrogen signalling also have interaction with the cytoskeleton (Kramár et al., 2009). Estradiol can activate a series of elements involved in actin signalling pathway, leading to actin filament assembly and enhanced synaptic response in hippocampal neurons (Kramár et al., 2009). The changes of cytoskeleton can not only induce morphological modifications of dendritic spines, but also affect protein trafficking. It has been revealed that the treatment with 17β-estradiol is able to modulate the trafficking of glutamate receptors in neurons, including the increase of NMDA subunit NR1 in dendritic spines and removal of AMPA receptor subunit GluR1 from dendritic spines to dendritic shafts (Srivastava et al., 2008). Since this trafficking of glutamate receptors occurs rapidly after 17β-estradiol treatment and does not depend on ERα activity (Srivastava et al., 2008), extranuclear ERβ should mediate this effect. This finding supports our view that ERβ can regulate protein trafficking, through which ERβ may accomplish its effects on late-LTP. According to the above-mentioned results and our findings, ERβ may contribute to the formation and maintenance of late-LTP through diverse ways. Its role appears to be quite complicated and needs more detailed investigations to be revealed.

5.5.3 The different sensitivity to ERβ between PSA- and fEPSP-LTP may be due to variant intracellular distribution and trafficking of proteins

Another remarkable finding in our studies on ERβ is that only PSA-LTP appeared to be sensitive to ERβ activity. Actually, the differences in the maintenance of PSA- and fEPSP-LTP have been reported before (Schulz and Korz, 2010b). In that study, it was found that PSA-LTP can be prolonged by water maze training, no matter in good or poor learners, whereas fEPSP-LTP was impaired in good learners, but reinforced in poor learners. Although the above-mentioned study used the cognitive reinforcement model, it indicates that PSA- and fEPSP-LTP does not share totally the same molecular mechanisms. From the cellular view, fEPSP is recorded in dendritic layer, whereas population spike is recorded in the DG granule cell layer. Therefore, the LTP of the fEPSP may reflect the compartment-specific plasticity within the dendritic layer, whereas PSA-LTP reflects the summed plasticity across distal and proximal compartments.

Previous studies revealed that late-LTP can be induced with a lower intensity of the tetanus in proximal compartments of the pyramidal neurons in the CA1 region than in the distal compartments (Sajikumar and Korte, 2011a,b), while the synthesis of PRPs such as protein kinase M zeta (PKMζ), which is synthesized after LTP-induction (Sacktor, 2008) and plays a critical role in LTP-maintenance (Sajikumar et al., 2005), have also been found to be activated by a weaker stimulation protocol in proximal than in distal compartments of the CA1 dendritic layer (Sajikumar and Korte, 2011a,b). These findings suggest that in different compartments of hippocampal dendrites, different mechanisms for plasticity are involved in the processing of incoming information. Late-LTP formation in the proximal compartments may require different PRPs in comparison to distal compartments. If the synthesis of these PRPs depends on the activation of ERβ and its target genes, shifts in the input summation by ERβ blockade would affect the late-PSA-LTP, but not necessarily the fEPSP-LTP, which is measured at a distinct location in the dendrictic layer. These differences between different dendritic compartments may be due to an asymmetric distribution of the molecules related to synaptic plasticity (both mRNA and proteins) from distal to proximal compartments. Since ERβ have been shown to be involved in the tranport of NMDA receptor and AMPA receptor subunits (Srivastava et al., 2008), we can speculate that ERβ may contribute to the distribution of PRPs into relatively proximal compartments of the dendrites in the DG (fig. 23). However, there are still no data proving the asymmetric distribution of the molecules

involved in ERβ functions in different compartments of dendrites. More studies are still required.

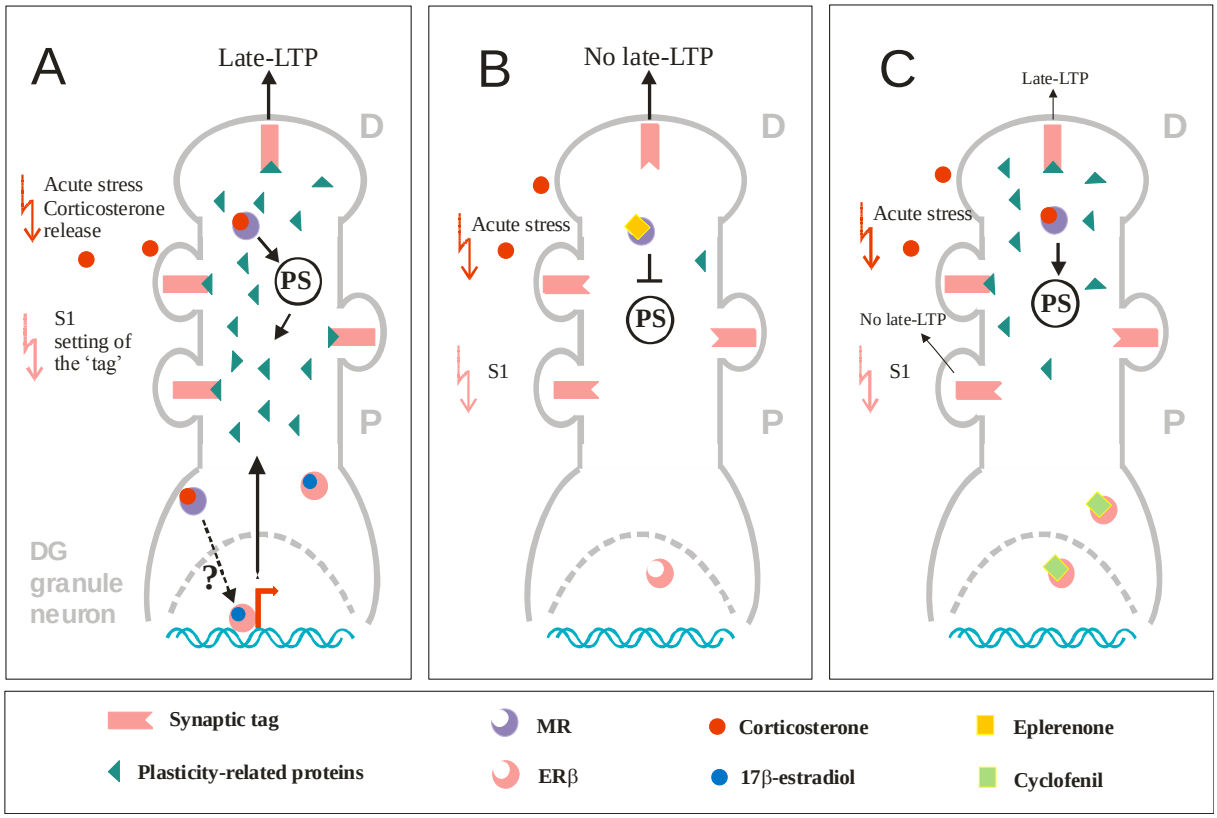

Figure 23. Proposed mechanism for signalling pathways involved inthe emotional LTP-reinforcement in the DG of the hippocampusbased on the 'synaptic tagging' theory. (A) After tetanus and a following acute stress, non-genomic MR is activated and then induces the local protein-synthesis of the PRPs specific for emotional LTP-reinforcement.The activated ERβ can additionally enhance the expression of these PRPs in a genomic way. Then, enough PRPs are produced for all 'tagged' synapses in the dendrites. The activated ERβ may also balance the distribution of the PRPs from distal to proximal compartments. Thereupon, late-LTP is formed in all compartments of the dendrites. (B) When MR activity is blocked by eplerenone, the majority of PRP synthesis can not be induced by tetanus/EPF-stress. Without PRPs, Late-LTP can not be formed in the 'tagged' synapses in all compartments of dendrites. (C) When ERβ activity is blocked by cyclofenil, MR activated by the acute stress can still induce the synthesis of the PRPs specific for emotional LTP-reinforcement partially. But these PRPs are not enough for all 'tagged' synapses. Without activated ERβ, proximal synapses may be at a disadvantage in the competition with distal synapses for the PRPs. The local synthesized PRPs in the dendrites may be inclined to distribute in relatively more distal compartments. Therefore, the limited PRPs just produce late-LTP in a part of the 'tagged' synapses, which are located in some relatively distal compartments. Accordingly, ERβ blockade can impair PSA-LTP-reinforcement, but not fEPSP-late-LTP.

5.5.4 The potential role of testosterone and AR in emotional LTP-reinforcement

Another interesting finding is that hippocampal testosterone levels were elevated by EPF-stress in MS+ rats, but not in MS- rats. Since this increase of testosterone level 1 h after post-tetanic stress was accompanied by failed LTP-reinforcement, it suggests that high level of testosterone in the hippocampus may negatively influence hippocampal plasticity. This finding is content with a previous observation that i.c.v. infusion of testosterone 30 min before tetanus can significantly impair cognitive (hole-board training) reinforcement of LTP in the DG *in vivo* (Schulz and Korz, 2010a). These effects of testosterone can be mediated by AR and ER (the latter can be activated by binding of testosterone metabolites) (Hojo et al., 2011) and probably accomplished by reduced activity of PRP.

Similar to estrogens, androgens can also induce rapid signalling in neurons (Hammes and Levin, 2007). A previous study found that acute injection of the anabolic steroid 19-nortestosterone acutely and transiently reduced the phosphorylation level of MAPK and NMDA receptor subunit GluN2B in rats' hippocampus, while the pretreatment with an AR antagonist prevented these effects (Rossbach et al., 2010). The phosphorylation of NMDA receptors is necessay for initiation and maintenance of LTP (Rosenblum et al., 1996; Rostas et al., 1996; Soderling and Derkach, 2000), whereas MAPK phosphorylation can be enhanced by exposure to acute stress (Korz and Frey, 2004) and is required for the formation of late-LTP (Reymann and Frey, 2007). Thus, high levels of hippocampal testosterone can impair LTP and even reduce baseline recordings of PSA (Schulz and Korz, 2010a). Although the present study did not reveal any changes in AR gene expression, the increased hippocampal testosterone level in MS+ rats can upregulate AR activity, which subsequently suppresses MAPK activation. This pathway may be one of mechanisms underlying the MS-induced suppression of emotional LTP-reinforcement.

In addition to the direct effects, testosterone can also be converted to estradiol by aromatase, which is expressed in the hippocampus (MacLusky et al., 1987; Hojo et al., 2004). This conversion has been found in the endoplasmic reticulumin cell body of neurons and in dendritic spines (Hojo et al., 2011). The produced estradiol then binds to ERs, driving a series of signalling pathways to regulate synaptic plasticity, which has been mentioned above. Since the testosterone in spines as well as the newly converted estradiol from testosterone are far from cell nuclei, the activated AR and ERs by them may mainly play non-genomic roles, whereas the testosterone and estradiol produced near the soma may mainly activate the genomic function of their receptors. This difference implicates another possibility to explain the different sensitivity to stimuli between different compartments of dendrites.

Actually, testosterone infusion also shows distinct effects on PSA and fEPSP in previous studies. Testosterone application reduces PSA-baseline, whereas it has no effect on fEPSP-baseline, but reinforces early LTP of fEPSP by in naïve animals (Schulz and Korz, 2010a). These findings may be associated to the different effects of ERβ blockade on PSA- and fEPSP-LTP. The interaction between androgen-signalling and ERβ in the hippocampus has been revealed, for instance, knockdown of ERβ, but not ERα, can attenuate the enhancement of avoidance learning induced by intrahippocampal infusion of testosterone (Edinger and Frye, 2007). On the other hand, the application of ERβ selective agonists can revert the learning deficit caused by androgen deprivation (Lagunas et al., 2011).

These evidences proved the involvement of ERβ in the signalling pathways activated by androgens in the hippocampus. This involvement may be achieved by the conversion from testosterone to estradiol as well as by other metabolites of testosterone and suggests that the signalling cascades induced by ERβ activation may share some common factors with androgen pathways to regulate hippocampal synaptic plasticity. Our results have offered more clues to reveal the interaction between androgens and estrogens as well as their receptors in the regulation of hippocampal synaptic plasticity.

5.6 The interaction between MR and ERβ

One of the most remarkable finding of the present study is that adult stress upreglated ERβ mRNA level in the hippocampus. Previous studies have not found direct evidences that stress hormones and their receptors can affect the gene expression of ERs. However, we have not found a direct evidence for the relationship between MR activity and *Esr2* expression. Our results only showed that the acute blockade of MR induced a tendency of decreased ERβ mRNA level contrasted to vehicle controls. But this tendency may suggest that MR may be partly involved in the mediation of the stress-induced upregulation of ERβ. It has been reported that the inhibition of MAPK and c-Src suppresses 17β-estradiol-induced gene expression of ERs in the cell culture of human breast cancer cells (Madak-Erdogan et al., 2008), while non-genomic MR is proved to mediate the aldosterone-induced rapid activation of MAPK (Grossmann et al., 2010) and c-Src (Braun et al., 2004). Although these results were mainly gained in studies with cell cultures, they supply us with the possibilities that the activated extranuclear MR by acute stress can also enhance ERβ gene expression through MAPK pathway in the hippocampus. On the other hand, acute stress not only activates MR, but also a series of other molecules and signalling pathways. For instance, serotonergic modifications are also found to be necessay for the emotional LTP-reinforcement (Ahmed et

al., 2006). The cascade activated by 5-hydroxytryptamine (5-HT) receptors, which can activate transcription factors like CREB (Meyer and Haebner, 1993; Ghosh and Greenberg, 1995; Nibuya et al., 1996), may be also involved in the regulation of ERβ gene expression. Therefore, acute stress in adulthood is able to induce ERβ gene expression with multiple mechanisms in certain tissues of CNS, including the hippocampus.

In addition, our results provide an evidence for the involvement of ERβ in the regulation of MR-mediated signalling of stress hormones, regulating hippocampal synaptic plasticity. Previous studies have showed a lot of results on the sex differences in the cognitive and behavioral responsiveness to stress (Luine et al., 1994; Galea et al., 1997; Wood and Shors, 1998; Bowman et al., 2001, 2003). But the role of sex hormone receptors in these sex-dependent effects was still not clear. The present study indicates that the MR-mediated hippocampal LTP-reinforcement of PSA depends on the gene expression and activation of ERβ. Since the blockade of ERβ did not affect MR gene expression after stress, the main role of ERβ in emotional LTP-reinforcement should be the interaction with the down-stream factors in the cascades activated by MR, but not the direct regulation of MR expression. These results indicate the importance of ERs as well as the interaction between MR and ERs in emotional memory processing even in males.

5.7 The behavioral and cognitive effects of MS

As discussed above, MS induced a repression of hippocampal MR and ERβ gene expression in response to stress. Chronic corticosterone injection (once per day for 21 consecutive days prior to the behavioral testing) has been found to increase depression-like behavior (Kalynchuk et al., 2004; Gregus et al., 2005; Johnson et al., 2006), whereas the treatment with 17β-estradiol shows an anxiolytic-like effect, which is ERβ-dependent (Walf et al., 2008). Associating our gene expression analysis to these previous findings, we strongly speculated a difference in anxiety-like behavior. Elevated plus maze (EPM) is a behavioral test widely used to analyze anxiety-like behavior of rodents (Walf and Frye, 2007). This test assesses anxiety-like behavior by ratio of time spent on the open arms to the time spent on the closed arms (Montgomery, 1958; Walf and Frye, 2007). Velocity and path length reflect the locomotion of the animals as additional assessment for anxiety responses.

A test/retest protocol was employed in the present study. Most results obtained with this approach showed increased open-arm avoidance in the second trial (Lee and Rodgers, 1990; Almeida et al., 1993; Griebel et al., 1993; Treit et al., 1993; Rodgers and Cole, 1994; Fernandes and File, 1996; Bertoglio and Carobrez, 2000). Our results are constistent with this

phenomenon. Both groups of rats spent more time in the closed arms in trial 2 than in trial 1. This phenomenon is usually explained by emotional memory formation. The animals that have undergone the initial exploration on the plus maze will acquire and consolidate some kind of memory related to the danger of the exploration in the open arms (Carobrez and Bertoglio, 2005). In the second trial, this memory will be retrieved (Carobrez and Bertoglio, 2005).

Although our MS+ and MS- rats did not differ in duration in either open or closed arms, the duration of MS+ rats in the center of the EPM apparatus was significantly beyond the random distribution in this area in the second trial of test, whereas this phenomenon was not detected in MS- rats. This result suggests that early-stressed rats may have a higher motivation to try the open arm, although usually they did not actually implement this action. If the decreased exploration in the open arms in trial 2 can be interpreted by the emotional memory formed in trial 1, the higher "motivation" to the exploration in the open arms implies a lower ability of acquirement, consolidation and/or retrievement of emotional memory. This decreased ability is likely due to a hypoactivity of the HPA axis in early-stressed rats, according to the observed lower level of corticosterone in MS+ rats. It has been reported that increased corticosterone level in the bed nuclei of stria terminalis (BNST) and the amygdala can reinforce anxiety-like behavior (Shepard et al., 2009; Myers and Meerveld, 2010). Although the difference in hippocampal corticosterone level between MS+ and MS- rats was only slight in our assays, the significant lower level of corticosterone in serum was suggestive of a probably lower corticosterone level in the BNST and amygdala. This attenuated hormonal responsiveness to stressors coincides with the mechanism underlying the attenuation of stress-induced LTP-reinforcement, further supporting our view that chronic MS paradigms at the end of SHRP is able to passivate the hippocampal sensitivity to stressful stimulations in adulthood.

5.8 Outlook

Based on the above-mentioned findings, the present study does not close a door, but open new windows for future researches. First of all, our MS paradigm is rarely used before. Now its significance for rats' neurobiological development has been partly revealed and can be used in future studies on the relationship between early-life stress, epigenetic mechanisms, synaptic plasticity and behavioral disorders. Although this study revealed some effects and underlying mechanisms, more investigations still should be performed on this MS paradigm.

5.8.1 Further research on MS-induced changes of gene expression in different subregions of the hippocampus and in different cell types

As a basic study, our research project has revealed that MS can repress stress-induced upregualtion of hippocampal MR and ERβ gene expression in adulthood in the whole hippocampus. Future studies should include the analyses of subregion and cell specific expression pattern of MR and ERβ genes. Since neurons, interneurons and glia play different roles in hippocampal plasticity, it is necessay in the future to analyze gene expression separately in different cell types so as to reveal how MR and ERβ contribute to hippocampal synaptic plasticity through the interaction between different cell types as well as how MS affect gene expression in various types of neural cells in the hippocampus.

In addition to the hippocampus, other brain regions also play critical roles in stress-induced LTP modifications, such as prefrontal cortex (PFC) and amygdala. MS may affect gene expression of steroid hormone receptors in these brain structures in a different way as the hippocampus. Thus, analyses for steroid hormones and gene expression of their receptors will also be performed in PFC and amygdale of both MS+ and MS- rats. Once differences in gene expression are found between groups, agonists or antagonists of the relevant receptors will be applied to observe their effects on emotional LTP-reinforcement. Moreover, PVN plays a key role in controlling HPA-activity and express high levels of AR and ERβ (Shughrue et al., 1997; Alves et al., 1998; Greco et al., 2001). The expression of gonadal steroid hormone receptors will be intensively analyzed in this region.

5.8.2 Further research on MS-induced epigenetic mechanisms

We found that our MS paradigm can induce changes of DNA methylation level in ERβ gene promoter region confronted by adult stress. But since our research revealed that MS induced decreased DNA methylation and repressed expression of *Esr2*, further studies have to perform to explain these observations. Fortunately, we have identified that the most CpG sites with different methylation level between MS- and MS+ rats are localized in cluster ADS108 from -329 to -118 in relation to the TSS. Thus, this sequence will be a crucial target for future studies. Namely, this finding offers the interpretation that under our conditions an “active” control of transcription factor binding and subsequent fine tuning of gene expression may play a role, in order to regulate emotional information processing and memory formation. Chromatin immunoprecipitation (CHIP) will be applied to search for the transcription factors binding with this sequence and investigate how DNA methylation impacts on the binding of relevant proteins.

As funds were limited, the present study only analyzed DNA methylation on *Esr2*. But it is likely that our MS paradigm can also induce epigenetic modifications in other genes, especially the genes encoding the enzymes directly involved in epigenetic mechanisms, such as DNMT, HDAC genes etc. So in the future, DNA methylation assays will be applied on more genes. Although we did not found the effects of pharmacological treatment with HDAC inhibitor, the roles of histone acetylation and other epigenetic mechanisms in MS-induced alterations in this study cannot be ruled out. Another research group in our institute using a similar MS paradigm in mice (from PND 14 to 16 3 h per day) has found a MS-induced increase of histone acetylation level around the promoters of Arc and EGR1 genes as well as decrease of protein phosphatase 1 (PP1) level (Not published data). It has been reported that PP1-catalyzed histone dephosphorylation is required by DNA methylation in some loci in the genome of *Neurospora crassa* (Adhvaryu and Selker, 2008).

Although the alteration of DNA methylation level has been verified, the whole process and mechanisms of this effect are largely unknown. Since there was no difference in basic level of methylation, it is also meaningful to reveal whetherthe activity of some methylation-related enzymes is altered by acute adult stress and whether this alteration shows differences between early-stressed animals and controls. Moreover, the separation paradigm on mice from PND 14 to 16 for everyday 3 h found alteration of histone acetylation level just 30 min after the last episode of MS (Not published data). Therefore, it is necessary in future studies to monitorthe changes of DNA methylation level of various genes at different time points after MS.

5.8.3 Further study on the role of ERβ in hippocampal synaptic plasticity

Pharmacological infusion with the antagonist of ERβ has proved the crucial role of ERβ in the maintenance of stress-induced late-LTP. The genomic actions as a transcription factor should be the main role of ERβ in the emotional LTP-reinforcement. To verify the genomic function of ERβ, future studies will perform immunohistological staining for ERβ so as to determine the subcellular location of this protein 1 h after tetanus/EPF-stress.

The up- and down-stream factors involved in the function of ERβ function will also be determined in next steps. The critical protein related to genomic ERβ functions may be 5-HT receptors. The activation of 5-HT receptors has been proved to be necessary for stress-induced late-LTP (Ahmed et al., 2006), while ERβ can enhance the gene expression of 5-HT_{2A} receptor (Sumner and Fink, 1993, 1995; Fink and Sumner, 1996; Cyr et al., 1998). Therefore, the relationship between 5-HT level and ERβ gene expression in the hippocampus as well as its role in emotional hippocampal plasticity also has to be revealed in the future.

Similar to 5-HT receptors, DAT gene expression can also be regulated by estrogen-involving pathways (Bossé et al., 1997), while the activation of dopamine receptors D1/D5 can enhance LTP expression in the DG (Kulla and Manahan-Vaughan, 2000). Thus, it is meaningful to verify whether the interaction between ERs and DAT exists in the hippocampus and plays a role in emotional LTP reinforcement in future studies.

Not only gene expression, previous studies also indicated some down-stream proteins whose activity and/or synthesis can be regulated by activated ERβ, including CREB (inceased phosphorylation level), AMPA receptor subunit GluR1 and PSD-95 (increased expression level). These effects are accompanied by enhanced LTP *in vitro* (Liu et al., 2008; Waters et al., 2009). But it still needs to be verified whether the ERβ-dependent upregulation of GluR1 and PSD-95 levels are also required for emotional LTP-reinforcement *in vivo*, because the present study did not observe a significant influence of ERβ level and activity on LTP maintenance in dendritic layer (fEPSP-LTP), whereas *in vitro* studies showed enhanced fEPSP-LTP, which was paralleled by increased dendritic branching and changes in spine morphology induced by the treatment with ERβ-agonists (Liu et al., 2008). Therefore, proteomic assays will be applied in the future to scan the target proteins of ERβ on their levels and activity in the hippocampus under different conditions of ERβ activity, so that more down-stream factors of ERβ pathways can be identified.

5.8.4 Further study on the interaction between MR and ERβ

The present study indicated a stress-induced upregulation of ERβ gene expression. But the mechanisms underlying this upregulation are still not clear. MAPK activation induced by extranuclear MR is speculated to play critical role. Thus, proteomic assays will be performed to analyze the modifications of MAPK as well as c-Src after acute stress in adulthood and verify whether the blockade of MR can affect the level and/or modification of these two proteins. On the other hand, MAPK and c-Src inhibitors will be applied in the future to verify whether their inhibition canimpair the upregulation of ERβ gene expression. In the same way, whether the level and activity of ERβ can modify the effects of MR on c-Src and MAPK activity is also need to be investigated in future researches. In addition, MR-blockade only induced a tendency of reduced ERβ gene expression, suggesting the involvement of other pathways in the upregulation of ERβ gene expression in response to stress. Moreover, GR may also be involved in the regulation of ERβ gene expression. Therefore, the infusion of GR antagonists will also be performed in the future.

On the other hand, the present study found that early-stressed rats showed a lower level of serum corticosterone after the second trial of EPM than control rats. The mechanisms underlying this difference in HPA axis need to be revealed in the future. Since the sensitivity of hippocampal ERβ gene expression to MS, we speculate the involvement of ERβ in this disorder of stress-responsiveness of MS+ animals. ERβ has been found to express in a high level in the PVN (Shughrue et al., 1997; Alves et al., 1998; Greco et al., 2001), which plays a key role in controlling the functions of HPA axis. Therefore, the gene expression level of ERβ in the PVN will be compared between MS+ and MS- rats after EPM and EPF-stress in order to reveal the mechanisms underlying the effects of MS on HPA axis in adulthood.

5.8.5 Further research on MS-induced behavior disorders

The main object of the present study is the functional interconnection of cellular molecular and behavioral in the regulation of emotional information processing. The behavioral part, however have to be extended. A variety of behavioral tests that can reveal different components of anxiety, motivation and cognition have yet not been introduced. However, we have found some implications for the effects of MS on anxiety-like behavior. As a rarely used model, our MS paradigm appeared to show an inverse effect on the risk of depression in adulthood compared to most MS paradigms, which increase anxiety-like behavior (Marco et al., 2009). Therefore, it is necessary to perform more behavioral tests to confirm the effects of our MS paradigm and investigate the mechanisms underlying these effects. Since different behavior between MS+ and MS- rats always appeared in the second trial in our EPM tests, we speculate that early-life stress may impair or actively inhibit the ability of emotional memory formation in the MS+ animals. Such kind of deficits of memory formation has been reported in the studies with fear conditioning (Wilber et al., 2009). Moreover, previous studies also showed dysfunctions of spatial learning with T-maze, Morris water maze and novel object recognition test (Garner et al., 2007; Aisa et al., 2007).

In addition, learning tasks like hole-board training and Morris water maze can also induce LTP-reinforcement (Korz and Frey, 2004; Uzakov et al., 2005; Schulz and Korz, 2010a,b). Because it has been shown that MR plays a regulatory role also in these tests, we can use the tests in the future, to assess possible effects of MS on cognitive reinforcement of LTP and memory by MS-induced modifications of learning and memory formation.

5.9 Summary and Conclusions

The present study verifies that postnatal stress is able to modulate *Esr2* DNA methylation pattern and gene expression in response to emotional challenges in adult male rats. Both kinds

of estrogen receptors as well as corticosterone binding receptors are expressed in the DG of adult male rats (Weiland et al., 1997; Kalita et al., 2005).

The specific activation of ERβ, however at present, cannot be explained. Synergistic as well as antagonistic models of the two estrogen receptor functions have been suggested (Tetel and Pfaff, 2010), changing in dependence of the internal and external situations (Neese et al., 2010). Although at present, there is no evidence for a direct activation of ER gene expression by corticosteroid hormones and their receptors, interactions between stress and sex hormones are common, and may constitute a complex network of steroid receptor effects and mechanisms that in concert mediate emotional memory consolidation (McEwen, 2002, 2010). In general, the present results suggest placing more emphasis on estrogen receptor modulation during stress studies even in male subjects. Especially, ERβ is critically involved in emotional reinforcement of hippocampal LTP and possibly in emotional memories.

Additionally, the MS paradigm used in the present study showed particular effects on HPA- and HPG-axis as well as hippocampal plasticity. Its effects on gene expression and DNA methylation did not appear in basal conditions, but only emerged in response to adult stress. The future studies on these particular effects may open the door to more epigenetic mechanisms in response to emotional challenges.

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Appendix: Information on DNA methylation assays for rat *Esr2* gene

A1.Rat *Esr2* promoter region and surrounding genomic DNA sequence

agcactggctgctcttccagagattctgagttcaaatcccagcaaccacatggtggcttgcaaccatct
gtaatgggatccggtgccctcttctggtgtgtctgaagacagctacagtgtactcattaaataaatctt
taaaaaaaaaaaaagtagatctttgaaacccttcataaacgccccatgatgtgtgcggatggcctgacc
actaattaggtacacgagtgacaccaggtcggcctacaggtcatttgttttccttttaacttttgccac
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gcattcaaacctccgcctccagcccttgcctttctggggctcaagctaacactccctggaaagggatct
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tccgttagtcGTGGGTAATCTTTGCAGCTTCTCCAGCTGCTGGCCTTTTTGAAACGCACTCTCAGGTCC
CTGCCTTCAGCGAGGCTTCTAGAATCAGCCACCTCTTGAAACTTCTTGGTGGGGAGCTGGCCCAGGGGG
AGCGGCTGGTGCTGCCACTGGCATCCCTAGGCACCCAGGTCTGCAATAAAGTCTGGCAGCCACTGCATG
GCTGAGCGACAACCAGTGGCTGGGAGTCCGGCTCTGTGGCTGAGGAAAGCACCTGTCTGCATTTAGAGA
ATGCAAAATAGAGAATGTTTACCTGCCAGgtaagtgtccctttgctcattgtgaggcttaattgaattt
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tgcccctagctaaaatgaatatgtcttagtcactctggcagcttgaactaaccagacatcgtttgcttt
cctcTGCAGTCATTACATCTGAGTCCCATGAGTCTCTGAGAACATAATGTCCATCTGTACCTCTTCTCA
CAAGGAGTTTTCTCAGCTGCGACCCTCTGAAGACATGGAGATCAAAAACTCACCGTCGAGCCTTAGTTC
CCCTGCTTCCTATAACTGTAGCCAGTCCATCCTACCCCTGGAGCACGGCCCCATCTACATCCCTTCCTC
CTACGTAGACAACCGCCATGAGTATTCAGCTATGACATTCTACAGTCCTGCTGTGATGAACTACAGTGT
TCCCGGCAGCACCAGTAACCTGGACGGTGGGCCTGTCCGACAGAGCACAAGCCCAAATGTGCTATGGCC
AACTTCTGGGCACCTGTCTCCTTTAGCGACCCATTGCCAATCATCGCTCCTCTATGCAGAACCTCAAAA
GAGTCCTTGGTGTGAAGCAAGATCACTAGAGCACACCTTACCTGTAAACAGgtaagtcttgtaaatcat
gggtggtggcatgctcctctctttgcaggttagtacgaaggaagacgtaagtaaaggtttttacttggc
c

i. Lower cases letters in green are 5' upstream sequence.

ii. Capital letters in purple are the exon 1 (5'UTR) sequence based on GenBank Accession# DQ273589.

iii. Lower case letters in blue are the intron sequence.

iv. Capital letters in black are the exon 1 coding region

v. CpG sites in this sequence are red (CGs).

vi. Green and yellow highlighted (G) is the transcriptional start site based on GenBank Accession#DQ273589.

vii. Green highlighted (C) is the translational start site based on Ensemble transcript ID: Transcript ID: ENSRNOT00000042682

viii. Green highlighted (ATG) is the translational start site (ATG).

ix. ADS110 target CpG loci are purple highlighted (CG).

x. ADS109 target CpG loci are turquoise highlighted (CG).

xi. ADS108 target CpG loci are blue highlighted (CG).

xii. ADS107 target CpG loci are yellow highlighted (CG).

xiii. ADS106 target CpG loci are gray highlighted (CG).

A2. Assay target sequences

Assay ID	Genomic Target Sequence	Bisulfite Converted Target Sequence	Pyrosequencing Dispensation order
ADS110FS	CTGGAGGCGGAGGTTTGAATGCATG TCTACCAGCGGCTGGCGATGGAAGT GGAGAGAAAAAAAAACAACGAGGGA	TTGGAGGYGGAGGTTTGAATGYATG TTTATTAGYGGTTGGYGATGGAAGT GGAGAGAAAAAAAAATAAYGAGGGA	CTGATGTCGAGTGATA GTCGTGTATATGTCGT AGTCGATGAGTGAGA GAATGATCGA
ADS109FS1	tgcgtgcgtccagggaccacacttc atcttgtttccatactttgacccac gcgcgtgtca	ygtgygtttagggattatattttat tttgtttttatattttgatttaygy gygtgtta	GTCGTAGTCGCTAGAT ATATTATTGTTATATTG ATGATCTGTCAGTCGT G
ADS109FS2	ccaggcccgggttctggtaatagc acgaaataagcagtaataacggag t	ttaggtttygggttttggtaatagt atygaaataagtagtaataayggag t	TCATGTTCGTTGTATA GTGATCGATAGTAGTA TGATCGAG
ADS108FS1	TCACGTGACCACAAACCCGGAGCCG GAGATTTCAGGCACTCCCGCGCCGC GCAACCGGATCCACTGGATGTCGAC CTCCA	TTAYGTGATTATAAATTYGGAGTYG GAGATTTTAGGTATTTTYGYGTYGY GTAATYGGATTTATTGGATGTYGAT TTTTA	TCGATCGTGATATGAT CGATGTCGAGATTAGT GATTCAGTCTGTCAGT CGTGATCGATATGATA GTCGAT
ADS108FS2	CCCCCTTCCAAGAGACTGACGATGG AGAACCGAGATGGAGCCCGGG	TTTTTTTTTAAGAGATTGAYGATGG AGAATYGAGATGGAGTTYGGG	ACTTAGAGATGTATCG ATGAGTATCGAGATGA TGTC
ADS107FS	AGAAGCCTCGCTGAAGGCAGGGACC TGAGAGTGCGTTTCAAAAAGGCCAG CAGCTGGAGAAGCTGCAAAGATTAC CCACGACTAACGGATGTTAGTGCGT CTTTTAAAG	AGAAGTTTYGTTGAAGGTAGGGATT TGAGAGTGYGTTTTAAAAAGGTTAG TAGTTGGAGAAGTTGTAAAGATTAT TTAYGATTAAYGGATGTTAGTGYGT TTTTTAAAG	GAGATGTTCGTGAGCT AGATGAGAGTAGTCG TTAAGTAGTAGTGAGA GTGTAGATATGATCGA TGATCGATGTAGTAGT CGTT
ADS106FS1	GAGCCGGACTCCCAGCCACTGGTTG TCGCTCA	AGTYGGATTTTTAGTTATTGGTTGT YGTTTA	AGATGTCGATCTAGTA TGTAGTCGTA
ADS106FS2	GATGCCAGTGGCAGCACCAGCCGCT CCCCCT	GATGTTAGTGGTAGTATTAGTYGTT TTTTTT	AGATGCTAGTGTCAGT ACTATGTCGT

A3. PCR protocol

ADS110, 109, 108, 107

Component	Per 30µl reaction
10X PCR buffer (Contains 15mM MgCl2)	3 µl (1x)
25 mM MgCl2	1.8 µl (3.0 mM final concentration)
10 mM dNTPs	0.6 µl (200 µM of each)
10 µM Fwd primer	0.6 µl (6 pmol)
10 µM Rev primer	0.6 µl (6 pmol)
HotStar Taq Polymerase (5 U/µl)	0.15 µl (0.75 U)
DNA	1 µl of bisulfite treated DNA
Water	Adjust to 30 µl

ADS106

Component	Per 30µl reaction
10X PCR buffer (Contains 15mM MgCl2)	3 µl (1x)
25 mM MgCl2	0 µl (1.5 mM final concentration)
10 mM dNTPs	0.6 µl (200 µM of each)
10 µM Fwd primer	0.6 µl (6 pmol)
10 µM Rev primer	0.6 µl (6 pmol)
HotStar Taq Polymerase (5 U/µl)	0.15 µl (0.75 U)
DNA	1 µl of bisulfite treated DNA
Water	Adjust to 30 µl

ADS110

95ºC 15 min; 45 x (95ºC 30s; **50.5**ºC 30 s; 72ºC 30 s); 72ºC 5 min; 4ºC

ADS108, 109

95ºC 15 min; 45 x (95ºC 30s; **60**ºC 30 s; 72ºC 30 s); 72ºC 5 min; 4ºC

ADS107

95ºC 15 min; 45 x (95ºC 30s; **62**ºC 30 s; 72ºC 30 s); 72ºC 5 min; 4ºC

ADS106

95ºC 15 min; 45 x (95ºC 30s; **54**ºC 30 s; 72ºC 30 s); 72ºC 5 min; 4ºC

A4.Pyrosequencing protocol

Pyrosequencing is performed using PSQ 96HS system or PSQ 96HSA system. The systems are equivalent to the PyroMark MD system. Pyrosequencing analysis is performed as per manufactory's recommended protocol with modification:

i. Prepare master Binding Solution:
   Components for 1 reaction of binding solution
   - 2.2 µl streptavidin
   - 40 µl of 2X Binding Buffer
   - 25 µl Milli-Q-water

ii. Add 65 µl of binding solution to each 15 µl PCR sample.

iii. Capture the PCR product as manufactory's protocol.

iv. Release the sepharose beads into annealing buffer containing 0.5 µM of a sequencing primer.

v. Anneal the sequencing primer to the template by heating the plate to 85 ºC for 2 minutes.

vi. Turn off the heating block and leave the Pyro plate on the heating block for 10 minutes.

vii. Remove the Pyro plate from the heating block and allow the plate continuing to cool for 5 minutes.

viii. Run the Pyrosequencing as the manufactory's instruction.

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Veröffentlichung	Wang, H., Meyer, K., Korz, V., 2012. Stress-induced Hippocampal mineralocorticoid and estrogen receptor β gene expression and long-term potentiation in male adult rats is sensitive to early-life stress experience. Psychoneuroendocrinology. In press. PubMed PMID: 22776422 http://www.ncbi.nlm.nih.gov/pubmed
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Selbständigkeitserklärung

Hiermit erkläre ich, Han Wang, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Msc. Han Wang

Magdeburg, 15.01.2012