# MOLECULAR MECHANISMS FOR REPROGRAMMING HIPPOCAMPAL DEVELOPMENT AND FUNCTION BY EARLY-LIFE STRESS

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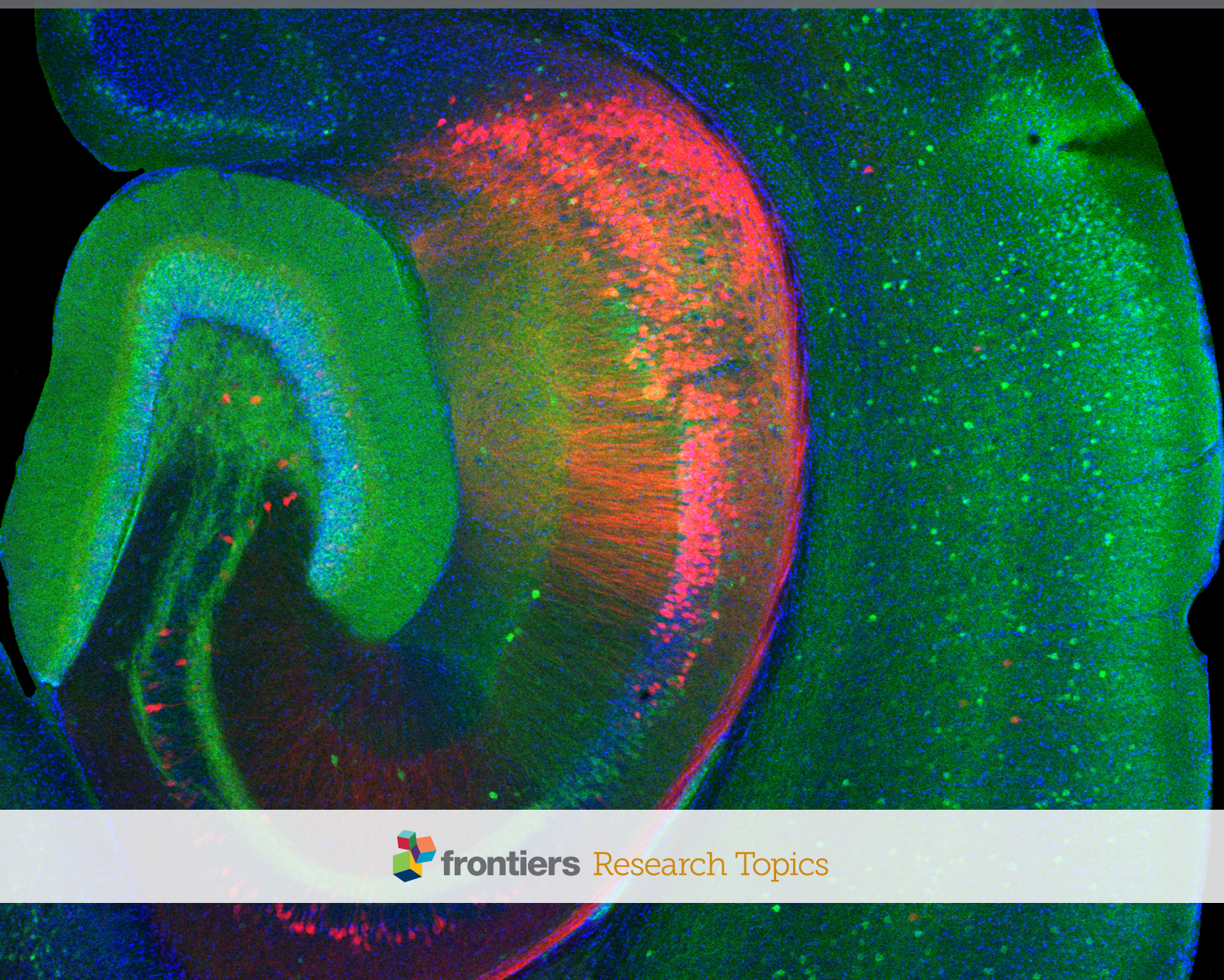

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# MOLECULAR MECHANISMS FOR REPROGRAMMING HIPPOCAMPAL DEVELOPMENT AND FUNCTION BY EARLY-LIFE STRESS

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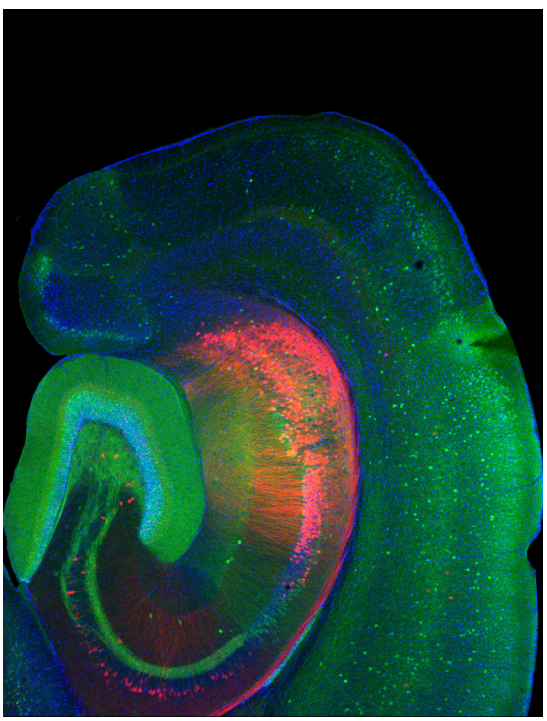

The mouse hippocampal formation. A subpopulation of excitatory and inhibitory neurons is marked by calbindin immunostaining (green). Pyramidal neurons in the subiculum, CA1 and CA3 that express Thy-1 cell surface antigen are shown in red. Nuclei are shown in blue. Photo by Xiao-Dong Wang.

The early postnatal period is a crucial stage for hippocampal development. During this critical period, the neonatal hippocampus is highly sensitive to the detrimental consequences of adverse environmental factors. Extensive clinical and preclinical evidence has shown that traumatic events early in life have profound and persistent effects on hippocampal function and behavior. This research topic focuses on the acute and lasting effects of early-life stress on various developmental

processes in the hippocampus, and aims to uncover the molecules that are responsible for early-life stress-programmed effects and underlie resilience or vulnerability to stress-related neuropsychiatric disorders later in life. We hope the articles in this research topic will provide novel insights and stimulate future studies on the mechanisms of early-life stress and brain development.

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EDITORIAL
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# Editorial: Molecular Mechanisms for Reprogramming Hippocampal Development and Function by Early-Life Stress

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**Keywords: early-life stress, hippocampus, development, plasticity, molecular mechanism**

**The Editorial on the research topic**

**Molecular Mechanisms for Reprogramming Hippocampal Development and Function by Early-Life Stress**

The hippocampal formation is both a key component of the medial temporal lobe crucial for declarative memory and a main target of stress mediators (e.g., glucocorticoids and neuropeptides) and stress-related molecules (e.g., nutritional factors and cytokines). During the first weeks of life, the hippocampus significantly increases in volume (Zhang et al., 2005) and several critical developmental processes coincide: generation of new neurons, outgrowth of neurites, formation of synaptic contacts, and establishment of neuronal circuits (Khalaf-Nazzal and Francis, 2013). Although the neonatal hypothalamic-pituitary-adrenal (HPA) axis is relatively hyporesponsive to environmental challenges, age-appropriate stressors can activate stress response, which in turn alters hippocampal development and increases the risk to develop neuropsychiatric disorders later in life, dependent on adult life conditions, and genetic predispositions (for recent reviews, see Lucassen et al., 2013; Tost et al., 2015; Bick and Nelson, 2016; Chen and Baram, 2016). As many neuropsychiatric disorders, such as schizophrenia and anxiety disorders, have developmental origins (Gross and Hen, 2004; Howes and Murray, 2014), dissecting the molecular mechanisms mediating the potentially detrimental consequences of early-life stress will provide insight into the pathophysiology and intervention of these disorders.

Most studies so far focus on the mechanisms of the long-term impact of early-life stress on hippocampal plasticity in adolescence/adulthood, which are of clinical relevance. In comparison, molecular mechanisms on how stress shapes the developing hippocampus have received attention only recently (Gross et al., 2012; Wei et al., 2012, 2015; Suri et al., 2013; Liao et al., 2014). We therefore initiated this research topic to sum up recent findings with an emphasis on both the dynamic effects of early-life stress on hippocampal structure and function at different life stages and the immediate effects of stress on hippocampal development.

Firstly, Huang provides an overview on the molecular and cellular alterations that modulate the effects of prenatal or postnatal stress on hippocampal development, and discusses how epigenetic modifications underlie the programming effects of early-life stress and contribute to the pathogenesis of epilepsy (Huang). In the dentate gyrus (DG) of the hippocampal formation, new neurons are continuously generated and selectively integrated to local circuits throughout the lifespan. Unlike the adult DG where most granule cells are mature and settled in place, during the first postnatal week the infrapyramidal blade of DG is yet to be formed and a majority of neurons are

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still immature. Exposure to severe stressors may thus perturb various aspects of neonatal and adult hippocampal neurogenesis and evoke lasting behavioral consequences. Lajud and Torner describe the key processes of neonatal DG development and review the short-term, intermediate and lasting effects of early-life stress on hippocampal neurogenesis (Lajud and Torner). Koehl further discusses the interaction between environmental factors (including early-life stress) and genetic background in shaping adult hippocampal neurogenesis and propose a conceptual framework for identifying genes that confer stress resilience or vulnerability (Koehl).

Early-life adversities are also manifested by malnutrition or infection. The disruption of maternal care inevitably alters the levels of nutritional and inflammatory factors in the offspring, which may modulate the influences of early stress. Hoeijmakers and colleagues present a comprehensive update on the intricate interplay among these essential elements of early-life environment and discuss their synergistic effects in shaping hippocampal structure and cognition, with a specific focus on adult neurogenesis (Hoeijmakers et al.). Moreover, Lardner summarizes our current understanding on the involvement of vitamin D, a vital nutrient with pleiotropic effects that may be insufficiently available under early-life stressful situations, in hippocampal development (Lardner).

Neurotrophins, especially brain-derived neurotrophic factor (BDNF), regulate neural circuit formation and activity-dependent synaptic plasticity via Trk receptors. Daskalakis and colleagues address the cross-talk between glucocorticoids and BDNF-TrkB signaling in early stress-induced hippocampal maldevelopment and behavioral deficits (Daskalakis et al.). In the research report by Wang and colleagues, BDNF protein level is examined in the hippocampus, medial prefrontal cortex and nucleus accumbens at different time points after neonatal maternal separation, and sex difference is further compared and discussed (Wang et al.). These two articles highlight the modulatory role of BDNF in early postnatal stress-programmed hippocampal development.

The serotonin (or 5-hydroxytriptamine, 5-HT) system is a main molecular target for the intervention of depression and anxiety and implicated in the acute stress response. In another highlighted research article, Bravo and colleagues evaluate the mRNA levels of two key components of the serotonin system, 5-$HT_{1A}$ receptor and serotonin transporter (SERT), in adult rats with or without a history of neonatal maternal separation, and find that early-life stress alters 5-$HT_{1A}$ and SERT mRNA levels in the amygdala and dorsal raphe nucleus, but not the hippocampus (Bravo et al.). These alterations may underlie the susceptibility of early-life stressed individuals to affective or anxiety disorders.

For altricial animals such as mice and rats, somatosensory input from the skin/whisker provides a major information source for representation of early-life environment. Erratic maternal care and/or peer interaction may thus result in abnormal experience-dependent synaptic plasticity and reshape the development of neocortex and hippocampus. Takatsuru and Koibuchi review how early-life stress disrupts the structure and activity of the somatosensory cortex and suggest the involvement of glucocorticoids, glutamate, and microglia in stress-induced somatosensory alterations (Takatsuru and Koibuchi).

Taken together, this research topic summarizes recent progress on the mechanisms of the effects of early postnatal stress on hippocampal development, and underlines the interactions of various factors in programming hippocampal plasticity. Meanwhile, many more interesting questions and new challenges emerge, some of which are sketched below.

1. **Dynamics.** Neural development and plasticity are highly dynamic, so are the influences of early-life stress. It is important to explore how stress dynamically modulates the levels and activity of stress-related molecules, and how these molecular events affect the dynamics of neuronal structure (e.g., formation and elimination of dendritic spines) and activity at different life stages.
2. **Interactions**. Future studies need to balance between addressing the complex interactions (e.g., between the timing and features of the stressor and concomitant critical developmental events; between genetic makeup and environmental elements; sex differences; etc.) and maintaining a manageable experimental design.
3. **Pathways.** Our understanding on the mechanisms of early-life stress may benefit from studies extending from dissecting the molecular pathways to mapping anatomical (i.e., neural circuits) and functional (i.e., network activity) pathways.
4. **Adaptation.** While early-life adversity is undoubtedly a major risk factor for adult pathologies, not all alterations resulting from early-life stress may be detrimental. Mounting evidence suggests that at least some of the molecular, structural or functional consequences of early-life stress exposure are adaptive and may increase individual resilience to similar challenges later on. Future studies will therefore also address how the observed alterations affect vulnerability or resilience to additional challenges in adulthood.

In short, we hope this collection will provide new perspectives and stimulate studies on the molecular mechanisms of early-life stress and brain development.

## AUTHOR CONTRIBUTIONS

XW and MS wrote the manuscript.

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# Early-life stress impacts the developing hippocampus and primes seizure occurrence: cellular, molecular, and epigenetic mechanisms

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Early-life stress includes prenatal, postnatal, and adolescence stress. Early-life stress can affect the development of the hypothalamic-pituitary-adrenal (HPA) axis, and cause cellular and molecular changes in the developing hippocampus that can result in neurobehavioral changes later in life. Epidemiological data implicate stress as a cause of seizures in both children and adults. Emerging evidence indicates that both prenatal and postnatal stress can prime the developing brain for seizures and an increase in epileptogenesis. This article reviews the cellular and molecular changes encountered during prenatal and postnatal stress, and assesses the possible link between these changes and increases in seizure occurrence and epileptogenesis in the developing hippocampus. In addititon, the priming effect of prenatal and postnatal stress for seizures and epileptogenesis is discussed. Finally, the roles of epigenetic modifications in hippocampus and HPA axis programming, early-life stress, and epilepsy are discussed.

**Keywords: early-life stress, epigenetic, epileptogenesis, hippocampus, hypothalamic-pituitary-adrenal axis, prenatal stress, postnatal stress, seizure**

## INTRODUCTION

The early-life environment is one of the most important factors affecting life-long health (Anand, 2000; van den Bergh et al., 2005; Lupien et al., 2009; Boksa, 2010; Strüber et al., 2014). In humans, early-life stress is associated with a preterm birth and a low birth weight, and can prime the neonate for further complications later in life that include psychiatric disorders, aged-related cognitive dysfunction, obesity, and hypertension (Barker et al., 1989; Fowden et al., 2005; Lemaire et al., 2006; Lahiri et al., 2009; Strüber et al., 2014). Animal studies also suggest that exposure to stressors or steroids during early-life alter the programming of the hypothalamic-pituitary-adrenal (HPA) axis, neurobehavior, and neuroimmune systems (Matthews, 2000; Mueller and Bale, 2008; Lupien et al., 2009; Brunton and Russell, 2010; Chen and Zhang, 2011; Lai and Huang, 2011; Strüber et al., 2014). Epigenetic modification has gained increasing attention in recent years because of its connection with early-life adversities (Weaver et al., 2004; Meaney et al., 2007; Mueller and Bale, 2008; Chen and Zhang, 2011; McClelland et al., 2011a,b; Murgatroyd and Spengler, 2011; Lucassen et al., 2013; Rabbe and Spengler, 2013). On the other hand, stress during development can have a significant epigenetic impact on the brain, and this relationship is bidirectional (Hunter, 2012).

Early-life stressors include prenatal, postnatal, and adolescence stress (Lupien et al., 2009; Schmidt, 2010). For example, in humans, early-life stress can include prenatal stressors such as exposure to exogenous glucocorticoids, maternal infection (King et al., 2005; Sørensen et al., 2009; Jenkins, 2013), and birth complications, as well as postnatal stressors such as exposure to exogenous glucocorticoids, maternal postpartum depression, loss of a parent, exposure to family conflict and violence, neglect, or physical maltreatment (De Bellis, 2002; King et al., 2005; Frodl et al., 2010). Both prenatal and postnatal stress can increase the likelihood of seizures in early life (Joels, 2009; Koe et al., 2009) and epileptogenesis in later life. This article focuses only on the influences of prenatal stress and postnatal stress.

## HIPPOCAMPAL AND HPA AXIS DEVELOPMENT

The hippocampus develops primarily during the fetal period in both rodents and primates (Seress et al., 2001; Khalaf-Nazzal and Francis, 2013). The limbic system, which includes the hippocampus, amygdala, and anterior cingulate cortex are already formed during the third and fourth month. Dentate gyrus forms at late stages of embryogenesis, however small numbers of dentate gyrus cells are formed from mid-embyrogenesis making temporal matching and connectivity of cells from other hippocampal subfields (Deguchi et al., 2011). Rodents and primates differ in the timing at which the majority of the dentate granule cells are produced; however, both rodents and primates produce ∼85% postnatally (Bayer, 1980a; Rakic and Nowakowski, 1981). A similar percentage of cornus ammonis (CA) 1–3 subfield neurons are produced during the last days of gestation in rodents, and during the first half of pregnancy in primates (Bayer, 1980b; Rakic and Nowakowski, 1981). The hippocampal subfields can be recognized with distinct molecular markers from embryonic stages (Khalaf-Nazzal and Francis, 2013).

In the rodent, maturation and full differentiation of the hippocampal formation takes place during early postnatal periods (Avishai-Eliner et al., 2002). During the first postnatal weeks, neuronal birth, differentiation, and migration are

ongoing (Altman and Bayer, 1990; Gould and Cameron, 1996). Neurogenesis of granule cells peaks during the second week of life in rodents (Bayer, 1980a), and during the third month in humans (Seress et al., 2001). In addition, synaptogenesis and the establishment of enduring connectivity patterns continue for weeks in the rodent, and for years in humans (Avishai-Eliner et al., 2002).

Glucocorticoids are released from the adrenal glands in response to stress, readily cross the blood-brain barrier, and activate hippocampal glucocorticoids receptors (McEwen, 1998). Glucocorticoids interact with their receptors in multiple target tissues, especially the HPA axis. Glucocorticoids act via two intracellular receptors, the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) to regulate gene transcription. In addition, glucocorticoids can change neural function via rapid nongenomic actions. GR and MR differ in ligand affinity and distribution (de Kloet et al., 2005): GR has a lower affinity than MR has, and therefore are more frequently occupied when corticosterone levels increase (de Kloet et al., 2005). The actions of glucocorticoids depend on the functionality of the balance between GR and MR in the brain (de Kloet et al., 2005).

There is a distinct ontogenic profile for GR and MR in the fetal rat brain (Diaz et al., 1998). GR mRNA is present in the anterior hypothalamus, hippocampus, and pituitary by gestational day 13 (Diaz et al., 1998), whereas MR mRNA is present in the hippocampus by gestational day 16 and the hypothalamus by day 17 (Diaz et al., 1998). GR and MR in the rat fetal brain are low throughout gestation, but increase rapidly after birth, consistent with the postnatal development of the brain in the rat (Diaz et al., 1998).

During pregnancy, the mother's HPA axis undergoes major changes (Lindsay and Nieman, 2005). Cortisol secretion increases steadily through gestation (Jung et al., 2011); thus, the normal physiological responses to stressors and the cortisol awakening response (i.e., basal HPA activity) are attenuated (Lindsay and Nieman, 2005). For most of the pregnancy, the baby and mother share a common corticotrophin-releasing hormone (CRH)-adrenocorticotropic hormone (ACTH)-cortisol axis (McLean et al., 1995).

By the end of the first week of life (Bohn et al., 1994; Vazquez et al., 1998), the number of MRs reaches adult levels. The number of GRs present during the first few week of life, however, is ∼30% of adult levels, but approach adult levels after ∼30 days of life. Both GR and MR are highly expressed in the developing brain, and have different and complex ontogenies that allow intricate brain development.

Between postnatal day 4 and 14, neonatal rat pups have low basal corticosterone levels and the corticosterone response to stressors is blunted, which constitutes the so-called stress hyporesponsive period (SHRP) (Levine, 2005). However, disruption of normal maternal behavior in rat during the SHRP can influence HPA axis development. In humans, the HPA axis is highly reactive and labile during early infancy, but organizes between 2 and 6 months of age through interactions between the infant and caregiver. The quality of caregiving that the infant receives predicts the infant's ability to self-regulate later in life. Sensitive caregiving is associated with better self-regulatory abilities and optimal functioning of the child's HPA system (Gunnar and Cheatham, 2002; Gunnar and Donzella, 2002).

## EFFECTS OF PRE-/POST-NATAL STRESS ON SEIZURE SUSCEPTIBILITY AND EPILEPTOGENESIS

Epileptogenesis is a process through which the normal brain develops epilepsy, and the hippocampus is implicated in the pathogenesis of both the initiation and propagation phases (Pitkänen and Lukasiuk, 2011). Mesial temporal lobe epilepsy (MTLE), the most common focal intractable epilepsy, is thought to be a multi-stage process of increasing epileptogenesis commencing in early life. The ongoing process of epileptogenesis and the course of epilepsy might be negatively influenced by the stress associated with the disease itself (Joels, 2009; Sawyer and Escayg, 2010). As a result, a negative loop might occur in which stress promotes epileptogenesis in predisposed individuals or lowers seizure threshold in epilepsy patients, thereby increasing the likelihood of exposure to stress, which in turn exacerbates the disease. Epidemiological data implicate stress in the cause of epilepsy and seizures in both children and adults (Temkin and Davis, 1984; Swinkels et al., 1998; Bosnjak et al., 2002).

Stress is a natural factor that may exacerbate or trigger seizures (Novakova et al., 2013; van Campen et al., 2013). HPA-related stress hormones, especially glucocorticoid and CRH, can affect excitatory and inhibitory processes in brain areas that are critically involved in seizure generation. Glucocorticoid exposure can alter plasticity in the hippocampus through increasing extracellular glutamate levels and calcium conductance (either voltage- or ligand-gated), alter expression of N-methyl-D-aspartate (NMDA) receptor subunits, and reduce glial uptake of glutamate, and thus, facilitate epileptiform discharges and seizures in animals. Glucocorticoids facilitate epileptiform discharges and seizures in animals. CRH is expressed in interneurons in both the developing and adult hippocampus and is released during stress (Sakanaka et al., 1987; Chen et al., 2001). Both glucocorticoids and CRH are important hormones that regulate the stress response and may contribute to seizure-induced loss of neurons, dendritic spines, and branching if it persists for a prolonged period (Ribak and Baram, 1996; Chen et al., 2012).

Negative life events and stress sensitivity are linked with childhood epilepsy (van Campen et al., 2012, 2013). In addition, epidemiological data implicate stress in the causation of epilepsy and seizures in children (Bosnjak et al., 2002). Specifically, early-life stress might create an enduring vulnerability to limbic epilepsy through altering glucocorticoids (Kumar et al., 2007), HPA axis (Joels, 2009), CRH (Baram and Hatalski, 1998), inflammation (Vezzani et al., 2013), membrane receptors such as gamma-aminobutyric acid (GABA) (Reddy, 2013), NMDA (Olney et al., 1991), and 2-amino-3-(3-hydroxy-5-methylisoxazol-4-yl) propionic acid (AMPA) receptors and neurotransmission (Rogawski, 2013), cellular electrophysiology, such as long-term potentiation (LTP) and long-term depression (Blaise et al., 2008), limbic area structures (Wong and Guo, 2013), and neuronal cell proliferation and neurogenesis (McCabe et al., 2001).

## PHYSIOLOGICAL MECHANISMS BY WHICH PRE-/POST-NATAL STRESS AFFECTS THE DEVELOPING HIPPOCAMPUS

### PRENATAL STRESS

#### *Glucocorticoid hormones*

During pregnancy, women have naturally elevated levels of cortisol. In general, normal glucocorticoid concentrations are essential for the development of several organs, including the central nervous system. Prenatal stress or synthetic glucocorticoid administration exposes the fetus to high glucocorticoid levels, which leads to downregulation of GR in the hippocampus, attenuation of negative feedback for the HPA axis, and enhanced HPA axis activity (Reul and de Kloet, 1985; Harris and Seckl, 2011).

#### *Placental CRH*

In humans, placental CRH activity is modulated by the maternal HPA axis (Wadhwa et al., 1998). Placental CRH concentration is a significant predictor of spontaneous preterm birth (Glynn et al., 2001; Sandman et al., 2006) and intrauterine growth restriction (IUGR) (Wadhwa et al., 2004), and can influence hippocampal development in the fetus. Prenatal stress activates the maternal HPA axis, which increases placental CRH production and its subsequent release into the bloodstream. A positive feed-forward loop between cortisol and placental CRH indicates that prenatal stress leads to progressively higher fetal plasma CRH levels. Placental CRH may penetrate the blood-brain barrier of the fetus, and subsequently influence both the function and the integrity of the hippocampus (Kastin and Akerstrom, 2002), presumably by activating CRH receptors (Sandman et al., 1999; Wadhwa et al., 2001).

#### *Placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2)*

The placenta is an effective barrier between the maternal and fetal hormonal environments in humans, being rich in 11β-HSD2, which converts cortisol to inactive cortisone (Benediktsson et al., 1997). Downregulation of placental 11β-HSD 2 increases glucocorticoid exposure for the placenta and fetus. Maternal stress not only increases her own circulating cortisol, it also reduces the expression and activity of 11β-HSD 2 in the placenta, leaving the fetus less protected (Avishai-Eliner et al., 2002; Mairesse et al., 2007). Moreover, inhibition of 11β-HSD2 might contribute to low birth weight, IUGR, and pregnancy disorders such as preterm birth and preeclampsia (Causevic and Mohaupt, 2007; Michael and Papageorghiou, 2008).

#### *Impaired uterine blood flow*

The impact of maternal anxiety on fetal blood flow can be determined by using ultrasound to measure the blood flow pattern in the uterine arteries. Sjostrom et al. found that, at 37–40 gestational weeks, mothers with high-trait anxiety scores had fetuses with higher indices of blood flow in the umbilical artery, and lower values in the fetal middle cerebral artery, suggesting a change in blood distribution that favored brain circulation (Sjöström et al., 1997).

### POSTNATAL STRESS

#### *CRH*

CRH is expressed in hippocampal interneurons and is released from axon terminals during stress. CRH is produced in several populations of cells in the developing hippocampus, such as Cajal-Retzius cells, and is involved in the maturation of hippocampal circuitry (Chen et al., 2001).

Chronic early-life stress, which was imposed by creating "simulated poverty" in the cage, resulted in cognitive problems and dendritic atrophy with loss of dendritic spines and synapses (Brunson et al., 2005). Many of the persistent effects of early-life stress are reversible with subsequent treatment with a CRH receptor 1 ($CRHR_1$) antagonist (Fenoglio et al., 2005). Adult mice lacking $CRHR_1$ in the forebrain were relatively resistant to the deleterious effects of chronic stress of social defeat (Wang et al., 2011a). Interestingly, the local deletion of $CRHR_1$ also protected adult mice from the adverse effects of chronic early-life stress on learning and memory (Wang et al., 2011b). Infusion of $CRHR_1$ antagonists immediately following this early-life stress prevented the learning and memory deficits, rescued LTP, and restored the integrity of the dendritic structure (Ivy et al., 2010). These findings provide direct evidence for a need for CRH-$CRHR_1$ signaling in the persistent effects of chronic early-life stress on hippocampal synapses. In this regard, Karsten and Baram propose that early-life experience can result in persistently altered regulation of CRH expression, which provides the neurobiological substrate to subsequent stress and some adult psychopathology (Karsten and Baram, 2013). In line with the preclinical data, single-nucleotide polymorphisms in the $CRHR_1$gene protect against depression in individuals exposed to childhood maltreatment (Tyrka et al., 2009).

#### *Glucocorticoid hormones*

Glucocorticoids are released from the adrenal glands in response to stress, readily cross the blood-brain barrier, and activate hippocampal glucocorticoids receptors (McEwen, 1998). Schmidt et al. demonstrated that glucocorticoid excess during the SHRP has only limited consequences on the adult behavioral phenotype (Schmidt et al., 2002). In addition, glucocorticoid administration early in life does not reproduce the effects of stress on hippocampal function and integrity when given in a non-stressful manner (Leverenz et al., 1999). Together, glucocorticoids play a minor role, and other factors may contribute more to the mechanisms by which early-life stress influences hippocampal development and function throughout life.

## PRENATAL STRESS

Prenatal stress is an important programming factor in brain development and function. A recent cross-sectional study indicated that 6% of pregnant women reported high levels of psychological stress during their pregnancies that resulted from conditions including depression, panic disorder, or domestic violence (Woods et al., 2009). Talge et al. reviewed several prospective studies related to prenatal maternal stress, and found a substantial number of emotional/behavioral problems in children, including attention deficit hyperactivity disorder, anxiety, and language delay, that were attributed to

prenatal stress or anxiety in ∼15% of the subjects (Talge et al., 2007).

## CELLULAR AND MOLECULAR ALTERATIONS IN THE DEVELOPING HIPPOCAMPUS THAT MAY LINK PRENATAL STRESS TO SEIZURE AND EPILEPTOGENESIS

### GLUCOCORTICOID AND CRH AND HPA AXIS

The density of hippocampal GRs was lower by ∼50% in prenatal stress female offspring; however, no difference was observed between prenatally stressed and control males (Szuran et al., 2000). This female-specific decrease in hippocampal GRs was also shown by Weinstock et al. (1992).

Szuran et al. restrained pregnant rat dams for 30 min/day during gestational days 15–19. Prenatally stressed females had higher basal corticosterone levels (Szuran et al., 2000). Exposure to exogenous glucocorticoids during the last week of gestation increased basal and stress-induced plasma corticosterone levels in adult rats (Seckl, 2004) and attenuated the HPA axis response (Seckl, 2004; Welberg and Seckl, 2001). Endogenous glucocorticoids mediated some of the changes in HPA responsiveness in prenatally stressed offspring, both in rodents and primates (Matthews, 2000).

### INFLAMMATION

Restrained pregnant mice dam offspring showed increased interleukin-1β and tumor necrosis factor-α level in the hippocampus, increased interleukin-1β immunoreactive microglial cells, and increased activated microglia. In addition, systemic administration of lipopolysaccharide induced a significant increase in tumor necrosis factor-α in the hippocampus of only prenatally stressed mice but not non-stressed animals (Diz-Chaves et al., 2012, 2013).

### MEMBRANE RECEPTORS AND NEUROTRANSMITTER

Maternal immune activation caused reduced basal neurotransmission of dopamine and glutamate, as well as reduced levels of the inhibitory transmitter GABA, within the hippocampus (Bitanihirwe et al., 2010). Prenatal stress also reduced the expression and activity of metabotropic glutamate receptor 5, which is implicated in the regulation of synaptic plasticity and neurogenesis in the hippocampus of male rats (Morley-Fletcher et al., 2011).

### CELLULAR ELECTROPHYSIOLOGY

A significant downregulation of hippocampal genes also was reported in 23-day-old female rats whose mothers were stressed from gestational days 17–21 (Bogoch et al., 2007). This included presynaptic voltage-gated $Ca^{2+}$ type P/Q and several $K^{+}$ channels that regulate the neuron membrane potential and suggests a potential decrease in the excitability of newly formed synapses.

### SPINE AND DENDRITE AND CELL MORPHOLOGY

Hayashi and colleagues reported that rats exposed to prenatal stress had a significant 32% reduction in synaptic density within the hippocampal CA3 area, as measured on postnatal day 35 (Hayashi et al., 1998). Lemaire et al. (2000) reported a reduction in the number of granule cells within the hippocampal dentate gyrus of prenatally stressed rats measured 28 days postnatally.

### NEURONAL CELL PROLIFERATION AND NEUROGENESIS

In male mice, prolonged prenatal stress decreased cell proliferation in the hippocampus by 60% on postnatal day 10 (Kawamura et al., 2006). In another experimental paradigm, daily maternal restraint during the last week of gestation resulted in deficits of hippocampal neurogenesis (Lemaire et al., 2006). The relationship between prenatal stress and neurogenesis is complicated and depends on the stressor type, sex, and environment. Prenatal stress seems to have both enhancing and suppressing effects on the development of hippocampal neurons in a stressor intensity-dependent manner (Fujioka et al., 2006). Fujioka et al. reported that short-lasting (i.e., 30 min, once daily, between gestation days 15–17) and mild prenatal stress seemed to enhance neonatal neurogenesis, facilitate LTP, and the differentiation of processes of hippocampal neurons, whereas long-lasting (i.e., 240 min, once daily, between gestation days 15–17) and severe prenatal stress impaired their morphology.

## EFFECTS OF PRENATAL STRESS ON SEIZURE SUSCEPTIBILITY AND EPILEPTOGENESIS

Beck and Gavin treated pregnant mice with beta-2-thienylalanine solvent or a sham injection on gestational days 10–12. Audiogenic seizures were tested on postnatal day 23. An increase in audiogenic seizure frequencies were observed in injected mice, irrespective of the nature of the injected substance. This finding suggested that the act of manipulation, rather than the test substance, caused stress and increased seizure propensity (Beck and Gavin, 1976). Frye and Bayon exposed rats to 20 min of restraint stress toward the end of their pregnancy (Frye and Bayon, 1999). They found that the prenatally stressed offspring had more partial seizures and tonic-clonic seizures with long durations than did control rats. Edwards et al. examined how stress exposure at different times during gestation might affect later limbic system excitability and the propensity to develop epilepsy (Edwards et al., 2002). Pregnant dams were restrained under bright light for 45 min, three times a day during either early gestation (gestational days 5–12) or mid-late gestation (gestational days 12–20). Offspring of the stressed dams were then tested as an infant at postnatal day 10 or as adults, and were compared with offspring from non-stressed dams. Outcome measures assessed were the stimulation-induced seizure threshold, after-discharge threshold, and the rate of seizure development using electrical hippocampal kindling. Both prenatal stressors significantly lowered after-discharge threshold in pups, but this effect appeared to diminish by adulthood in the early gestational stress group. In addition, mid to late gestational stress accelerated kindling rates in all infant offspring and in adult males, but had no effect in adult female rats. Notably, Young et al. administered dexamethasone or betamethasone on gestational days 15–18, and tested the seizure threshold and kindling parameters (Young et al., 2006). They found prenatal betamethasone treatment increased seizure threshold for both models. Prenatal dexamethasone treatment increased kindling threshold, but not seizure threshold. Kindling rate was unaffected by either glucocorticoid treatment (Young et al., 2006). Velisek showed prenatal exposure to betamethasone decreased postnatal susceptibility to flurothyl-induced clonic seizures but not to kainic acid-induced seizures. Prenatal hydrocortisone decreased

postnatal weight but did not affect seizure susceptibility (Velíšek, 2011). In their subsequent work, Yum et al. demonstrated that prenatal restraint stress (2 × 45 min) in rats on gestational day 15 would increase susceptibility to spasms on postnatal day 15 (Yum et al., 2012).

Shang et al. showed an association between the onset risk of infantile spasms and the degree of maternal stress (Shang et al., 2010). However, in a population-based cohort study in Denmark, Li et al. studied children who were hospitalized because of epilepsy and born to women who had lost a close relative during pregnancy 1 year before pregnancy (Li et al., 2008). In this study, no association was found between this particular form of prenatal stress and the risk of epilepsy.

Indirect evidence links prenatal stress and an increased likelihood of childhood seizures in children with autistic disorder. Minshew et al. pointed out that epilepsy is found in about one-third of patients with autistic disorder, a disorder related to prenatal stress (Kinney et al., 2008), compared with a prevalence of only 2–3% in the general population (Minshew et al., 2005). **Table 1** summarizes the current rodent studies regarding the impact of prenatal stress on seizure occurrence and epileptogenesis.

## POSTNATAL STRESS

Early-life adversity (childhood abuse and neglect, loss of parents, or extreme poverty) occurs worldwide and are all too common in the lives of children (Jones, 2008; Sandberg and Rutter, 2008). In the Dunedin Study birth cohort of 1037 children, followed prospectively for 32 years, maltreatment includes maternal rejection, harsh discipline, sexual abuse, physical abuse, and disruptive caregiver changes (Danese et al., 2009). For each child, the cumulative index counts the number of maltreatment indicators experienced during the first decade of life; 63.7% of children experienced no maltreatment, 26.7% experienced one form of maltreatment, and 9.6% experienced two or more forms of maltreatment (Danese et al., 2009). Clinical evidence from life-course epidemiology study points to the importance of early life experiences in shaping adult disease (Poulton et al., 2010).

## CELLULAR AND MOLECULAR ALTERATIONS IN THE DEVELOPING HIPPOCAMPUS THAT MAY LINK POSTNATAL STRESS TO SEIZURE AND EPILEPTOGENESIS

### GLUCOCORTICOID AND CRH AND HPA AXIS

A 24-h maternal separation paradigm in 11-day-old rat pups can lead to a decrease in the expression of GR and MR mRNA in the hippocampus (van Oers et al., 1998). Likewise, expression levels of GR and MR are down regulated in the hippocampus of maternally separated mice on postnatal day 9 (Schmidt et al., 2002). In addition, neonatal infection in mice led to altered hippocampal GR and MR mRNA, as well as proteins, following a subsequent adult infection (Wynne et al., 2011).

Wang et al. demonstrated that early postnatal life stress impairs hippocampus-dependent spatial learning and memory in adult mice, and is associated with physiological, morphological, and molecular abnormalities in the hippocampus (Wang et al., 2011a,b). Impairments of spatial learning and memory in early postnatal life stress are recapitulated by forebrain CRH overexpression and attenuated by forebrain $CRHR_1$ inactivation. This suggests the forebrain CRH-$CRHR_1$ system is crucial for modulating and programming cognitive functions by early-life stress (Wang et al., 2011a,b).

### INFLAMMATION

In rat, maternal separation on postnatal day 9 caused increased hippocampal interleukin-1 receptor in male offspring (Viviani et al., 2014). In the hippocampus, a decrease in BDNF mRNA and an increase in interleukin-1β mRNA were observed in rats with a neonatal infection and an immune challenge in adults (Bilbo et al., 2008).

### MEMBRANE RECEPTORS AND NEUROTRANSMITTER

Maternal separation on postnatal day 9 decreased the levels of the AMPA receptor GluA1 and GluA2 subunits, altered NMDA receptor subunits GluN2B to GluN2A ratio, and increased interleukin-1 receptor interactions with GluN2B at the synapse of male hippocampal neurons (Viviani et al., 2014). This mechanism is part of a complex re-organization of the excitatory glutamatargic synapses. Hsu et al. reported two episodes of handling with maternal separation during early postnatal development resulted in long-term changes in postsynaptic GABA receptor function and subunit expression in hippocampal dentate gyrus (Hsu et al., 2003).

### CELLULAR ELECTROPHYSIOLOGY

Maternal separation prevented the stress-induced transformation from early to late LTP in the dentate gyrus of adult male rats (Wang et al., 2013b). However, maternal separation for 24 h on postnatal day 3 facilitated LTP in the dentate gyrus after an acute stress (Oomen et al., 2010).

### SPINE AND DENDRITE AND CELL MORPHOLOGY

An altered granule cell dendritic morphology (Oomen et al., 2010), a lower number of hippocampal neurons and glia (Leventopoulos et al., 2007; Fabricius et al., 2008), and a reduced mossy fiber density (Hout et al., 2002) have been reported following maternal separation (Rodenas-Ruano et al., 2012). Wang et al. demonstrated that postnatally stressed adult mice had decreased hippocampal nectin-3 levels and dendritic spine loss via CRH mechanism (Wang et al., 2013a).

### NEURONAL CELL PROLIFERATION AND NEUROGENESIS

Maternal separation for 180 min leads to an increase in cell proliferation on postnatal day 21 (Nair et al., 2007); however, in 2- to 7-month-old rats, cell proliferation was reduced (Mirescu et al., 2004; Oomen et al., 2010; Hulshof et al., 2011).

Maternal separation for 24 h on postnatal day 3 increases hippocampal neurogenesis (Oomen et al., 2009). Similar to cell proliferation, early stress is associated with distinct consequences on hippocampal neurogenesis that manifest in a temporally regulated manner, i.e., enhanced in young adulthood and impaired in middle-aged (Suri et al., 2013).

## EFFECTS OF POSTNATAL STRESS ON SEIZURE SUSCEPTIBILITY AND EPILEPTOGENESIS

Edwards et al. investigated the effects of maternal separation on kindling epileptogenesis utilizing a relatively benign separation

**Table 1 | Summary of rodent studies investigating effects of prenatal stress in rodent models of epilepsy/epileptogenesis.**

| Author | Manipulation in prenatal life | Endpoint test of seizure threshold or epileptogenesis | Outcome measurements | Conclusions/implications |
|---|---|---|---|---|
| Beck and Gavin, 1976 | Pregnant dams received beta-2-theinylalanine or solvent on GDs 10–12 Control: unhandled mice | Audiogenic seizures on PND 23 | Increased seizure frequencies in injected mice, irrespective of the nature of the injected substance | Prenatal stress increased seizure susceptibility in young age |
| Frye and Bayon, 1999 | Maternal restraint stress of mother for 20 min on GD 18 Control: no restraint stress rats | Adult gonadectomized offspring were administered 3 alpha, 5 alpha-THP 1 h prior to testing for kainic acid-induced seizures | Increased seizure production and longer duration in stressed offspring Lower dose of 3 alpha, 5 alpha-THP was effective in reducing seizure duration in control females Higher dose of 3 alpha, 5 alpha-THP was needed to reduce seizure duration in prenatally stressed females and males | Prenatal stress decreases neurosteroid's anti-seizure capability. Effects are sex-dependent |
| Edwards et al., 2002 | Midde restraint stress (45 min, 3×/day, GDs 5–12) Late restraint stress (45 min, 3×/day, GDs 12–20) | ADT and Hippocampus kindling on PND 14 or in adults | Lowered ADT on PND 14 infant rat offspring in both early and late gestation stressed rats. Increased kindling rate in infant and adult male offsprings of middle and late gestation stress, but not in females. No effect on ADT | Prenatal stress, in particular during the latter half of gestation, increases seizure vulnerability in the unborn offspring. The offspring appear most susceptible to seizure development during the infantile period, but some effects persist into adulthood, particularly in males |
| Young et al., 2006 | Pregnant dams received once daily injections with dexamethasone (0.2 mg/kg/day) or betamethasone (0.2 mg/kg/day) between GDs 15–18 | Seizure thresholds were determined on PND 14 using electroconvulsive shock. Hippocampus kindling on PNDs 14–15 | Prenatal betamethasone increased seizure threshold for both models. Prenatal dexamethasone increased kindling threshold, but not electroconvulsive shock threshold. Kindling rate was unaffected by either prenatal glucocorticoid | Prenatal repeated glucocorticoid treatments raised seizure thresholds and reduced seizure vulnerability, seemingly "favorable" |
| Velíšek, 2011 | Pregnant dams received hydrocortisone (2 × 10 mg/kg) or betamethasone (2 × 0.4 mg/kg) on GD 15 | Seizures induced by flurothyl or kainic acid on PND 15 | Prenatal exposure to betamethasone decreased postnatal susceptibility to flurothyl-induced clonic seizures but not to kainic acid-induced seizures. Prenatal hydrocortisone did not affect seizure susceptibility | Prenatal exposure to glucocorticoids on seizure susceptibility may be seizure syndrome specific |
| Yum et al., 2012 | Prenatal restraint stress (2 × 45 min) GD 15 | Development-specific spasms triggered by NMDA on PND 15 | Prenatal stress significantly accelerated onset and increased number of NMDA-triggered spasms | Prenatal stress may enhance susceptibility to develop triggered spasms in infant rats. This finding is similar to increased risk for development of infantile spasms in children of mothers with gestational stress |

*ADT, afterdischarge threshold; GD, gestational day; NMDA, N-methyl-Daspartate 3 alpha; PND, postnatal day; 5 alpha-THP, 5 alpha pregnan-3 alpha-ol-20-one.*

protocol that included 60 min on postnatal days 4 and 5 (Edwards et al., 2002). The comparison group included the other littermates, which were briefly handled but not removed from the mother. This postnatal manipulation had no effect on after-discharge threshold or rapid hippocampal kindling rates when assessed at 2 weeks of age.

To investigate the effects of maternal separation on the long-term consequences of early-life status epilepticus, Lai et al. tested whether maternal separation for 1 h affected the long-term sequelae of emotional disorders following seizure early in life (Lai et al., 2006). Lai et al. used maternal separation that involved 1 h of isolation daily during postnatal days 2 and 9, and used lithium-pilocarpine-induced status epilepticus on postnatal day 10 rats. As adults, anxiety-related behavior was assessed using the elevated plus maze test and seizure susceptibility was assessed by pentylenetetrazol-induced seizures. Rats exposed to maternal separation and seizures demonstrated a reduced pentylenetetrazol threshold for seizure induction compared to non-handled rats or rats exposed to isolation or seizure alone. Metyrapone (a corticosterone synthesis inhibitor) treatment prior to seizure did not reverse this enhanced excitability, indicating a partial role of glucocorticoids in this context. Salzberg et al. examined the effects of maternal separation on limbic excitability and the development of amygdala kindling (Salzberg et al., 2007). Postnatal stress was induced by separating pups from their mothers for 180 min daily from postnatal days 2–14. The comparison condition was mother and pup separation for 15 min per day over the same period, an exposure referred to as early handling. At 8 weeks of age, equivalent to young adult life, rats were tested for the after-discharge threshold and subjected to rapid amygdala kindling. Rats exposed to early-life stress exhibited significantly lower seizure thresholds and an accelerated rate of kindling, compared to early handled rats. These effects on limbic excitability and epileptogenesis were specifically observed in female rats, whereas males did not demonstrate changes in epilepsy outcomes, despite demonstrating increases in anxiety-like behavior. Using the rat amygdala-kindling model, Kumar et al. demonstrated that early-life stress induced by maternal separation accelerates the progression of focal limbic seizures to secondary generalized convulsive seizures in adult rats (Kumar et al., 2011). Desgent et al. used a two-hit model of TLE characterized by two early-life insults: a freeze lesion-induced cortical malformation on postnatal day 1, and a prolonged hyperthermic seizure on postnatal day 10 (Desgent et al., 2012). They demonstrated that after both insults, females did not develop MTLE while all males did. This correlated with a rise in corticosterone levels on postnatal day 1 following the lesion, but only in males. Their data demonstrated sexual dimorphism in the long-term vulnerability for developing epilepsy in the lesion plus hyperthermia animal model of MTLE, and suggested that the response to early-life stress at postnatal day 1 contributed significantly to epileptogenesis in a sex-specific manner (Desgent et al., 2012). Ali et al. demonstrated changes in firing patterns in thalamocortical and hippocampal regions resulting from both maternal separation and amygdala kindling, which might reflect cellular changes underlying the enhanced vulnerability to kindling in rats that had been exposed to early-life stress (Ali et al., 2013).

Similarly, Leussis and Heinrichs cross-fostered El pups to CD-1 dams because CD-1 dams exhibit a higher quality of maternal care than El dams. El pups raised by CD-1 dams experienced delayed seizure onset and reduced seizure frequency, suggesting that early-life environment can play an important role in shaping the adult seizure phenotype (Leussis and Heinrichs, 2009). It should be noted the El mouse model has not been verified for its effect on early-life stress. In addition, El pups raised in a biparental environment with both the El dam and sire attending the pups received more parental attention than El pups raised by only the El dam, yet they showed an earlier development of seizures (Orefice and Heinrichs, 2008). Together, early-life environment can interact with a genetic predisposition to shape the future seizure phenotype.

Van Campen et al. studied the effect of stress on seizure frequency in childhood epilepsy. They found stress sensitivity was reported in half of the children with epilepsy. They suggested that experiencing negative life events might cause a larger response to daily stressors, thereby increasing the likelihood to induce epileptic activity in childhood (van Campen et al., 2012). **Table 2** summarizes the current rodent studies regarding the impacts of postnatal stress upon seizure occurrence and epileptogenesis.

## EPIGENETIC MODIFICATIONS IN DEVELOPMENT PROGRAMMING AND THE EFFECTS OF STRESS

Epigenetic modifications regulate gene expression without altering the DNA sequence. Epigenetic changes involve DNA methylation at cytosine-guanine sequences-CpG sites, histone posttranslational modifications (histone methylation, acetylation, phosphorylation, ubiquitylation, sumoylation, and propionylation), and microRNAs (Gräff et al., 2011). Epigenetic mechanisms control nucleosome spacing and how they are condensed, which subsequently determines gene activity. Briefly, chromatin exists in an inactivated and condensed state (heterochromatin) that prevents gene transcription, but when activated to an open state (euchromatin), genes can be transcribed.

It is now clear in both humans and animals that glucocorticois and stress have a significant epigenetic impact, and the relationship between the stress response and epigenetics in the brain is bidirectional (Hunter, 2012). Epigenetic alterations have become especially attractive to researchers in recent years, as increasing evidence indicates that they can be induced by physical and social exposure early in life (Meaney et al., 2007). For some neurobiological disorders, exposure to environmental agents during early developmental stages can epigenetically disturb gene regulation in a long-term manner and cause significant pathological manifestations later in life. This process is the latent early-life associated regulation model by Lahiri et al. (2009).

Epigenetic dysregulation has been associated with prenatal IUGR and disease in both humans and rodents (Baserga et al., 2007, 2010; Friso et al., 2008). Prenatal stress can cause increased DNA methylation in the frontal cortex and hippocampus (Mychasiuk et al., 2011; Matrisciano et al., 2013) and a lower DNA methyltransferase 3a immunoreactivity in the dentate gyrus in offspring (Sierksma et al., 2013).

**Table 2 | Summary of rodent studies investigating effects of postnatal stress in rodent models of epilepsy/epileptogenesis.**

| Author | Manipulation in postnatal life | Endpoint test of seizure threshold or epileptogenesis | Outcome measurements | Conclusions/implications |
|---|---|---|---|---|
| Edwards et al., 2002 | Maternal separation (1 h/day, PNDs 4–5) Control: non-stressed littermates | ADT and Hippocampus kindling on PND 14 | No effect on ADT or kindling rate | Postnatal stress has no effect on infant seizure susceptibility |
| Lai et al., 2006 | Maternal separation (1 h/day, PNDs 2–9) and SE induced by lithium-pilocarpine Control: normal rearing and SE induced by lithium-pilocarpine | pentylenetetrazole-induced seizures at PND 100 | Prolonged seizure duration and reduced seizure threshold following early life SE in stressed rats | Early life stress increases the vulnerability to seizures in adulthood |
| Salzberg et al., 2007 | Maternal separation (180 min/day, PNDs 2–14) Control: EH (separation 15 min/day, PNDs 2–14) | Rapid amygdala kindling on ∼PND 56 | Stress female rats had increased kindling rate and reduced seizure threshold; no differences in male | Early life stress contributes to epileptogenesis. Effects are sex-dependent |
| Orefice and Heinrichs, 2008 | Amount of parental care between PNDs 2–21 on genetically susceptible EI mouse seizure emergence | HISS test on PNDs 80–90 | HISS testing of adult EI offspring revealed a deleterious effect of biparental rearing as a second care provider is a stressor in EI pups | Early life stress increased seizure suscebtibility in adult EI mice |
| Leussis and Heinrichs, 2009 | Cross-fostering genetically susceptible EI pups to a seizure-resistant CD-1 mothers | HISS test on PNDs 80–90 | cfos hypoactivity in hippocampus and cortex on PNDs 35–40 as a result of HISS. EI mice offspring with improved maternal care showed delayed onset of HISS-induced seizure susceptibility on PNDs 80–90 | Increased maternal care in genetically susceptible EI mouse may have prophylactic benefits for neural plasticity and adult seizure susceptibility |
| Kumar et al., 2011 | Maternal separation (180 min/day, PNDs 2–14) Control: EH (separation 15 min/day, PNDs 2–14) | Rapid amygdala kindling on ∼PND 56 | Stress rats has accelerating kindling rates, enhanced corticosterone response to kindled seizure, decreased hippocampal pyramidal cell numbers, and enhanced kindling-induced neurogenesis in adulthood | Alternations of hippocampal pyramidal cell neurogenesis are candidate mechanisms that early life stress promotes vulnerability to epileptogenesis. Effects are sex dependent |
| Desgent et al., 2012 | Two early life insults: a freeze lesion-induced cortical malformation at PND 1 and a hyperthermic seizure at PND 10 | Video-EEG from PND 90 to 120 | Increased susceptibility to PND 10 hyperthermia-induced convulsion in PND 1 lesioned rat. Two hits in females did not develop mesial temporal lobe epilepsy while all males did | Early life stress contributes to epileptogenesis. Effects are sex-dependent |
| Ali et al., 2013 | Maternal separation (180 min/day, PNDs 2–14) Control: EH (separation 15 min/day, PNDs 2–14) | Amygdala kindling | Hippocampus: stress rats had more % APs firing in burst | Sress rats had enduring alterations in the firing patterns of neurons in the hippocampus that may underlie the increased vulnerability to limbic epileptogenesis |

*ADT, afterdischarge threshold; APs, action potentils; EEG, electroencephalogram; EH, early handling; HISS, handling-induced seizure susceptibility; PND, postnatal day; SE, status epilepticus.*

Variations in maternal care in the rat result in differences in hippocampal development and synaptic plasticity in the offspring (Macrì and Würbel, 2006). Observational studies provide evidence for two forms of maternal behaviors during the first week of lactation: licking/grooming (LG) and the arched-back nursing (ABN) posture (Liu et al., 1997; Francis et al., 1999). In the rat, the adult offspring of high LG-ABN mothers show increased hippocampal GR expression and enhanced glucocorticoid feedback sensitivity compared to animals reared by low LG-ABN mothers (Liu et al., 1997; Francis et al., 1999). In addition, adult offspring of high LG-ABN mothers exhibited modest HPA stress responses compared to animals reared by low LG-ABN mothers (Menard and Hakvoort, 2007). In hippocampus, offspring from high LG-ABN mothers had hypomethylation of CpG dinucleotides in the exon $1_7$ GR promoter sequence, and increased histone acetylation that might account for higher transcription of the GR gene (Weaver et al., 2004). The maternal effect is mediated by enhanced serotonergic activity and an increased expression of NGFI-A, which binds the exon $1_7$ GR promoter sequence (Weaver et al., 2007). Cross-fostering experiments showed a causal relationship between maternal care and changes in the exon $1_7$ GR promoter methylation (Weaver et al., 2005, 2006).

## EPIGENETIC MODIFICATION IS A SHARED PATHOGENIC SUBSTRATE OF BOTH EARLY-LIFE STRESS AND EPILEPSY

As stated above, epigenetic modifications underpin the programming effects of early-life stress. Interestingly, a wealth of evidence indicates that dysregulation of epigenetic mechanisms occurs in several human epilepsy syndromes. Epigenetic mechanisms can influence the acute deployment of genes resulting from seizures themselves or can have gradual effects on the steady-state expression profile of candidate genes that persist into epilepsy. Epigenetic modifications can affect seizure and epilepsy in several ways (Lubin, 2012; Roopra et al., 2012).

Firstly, histone acetylation is involved in epileptogenesis in human epilepsy patients. Seizure activity results in gene expression changes, including alterations in mRNA levels for glutamate receptor 2 and BDNF, the two well-characterized epileptogenesis-related genes. Of interest, histone acetyltransferase-mediated increases in histone acetylation levels at the promoter regions of the glutamate receptor 2 and BDNF genes have been shown to correlate with their gene expression changes following seizures in an experimental animal model (Huang et al., 2002b).

Secondly, DNA methylation has been highlighted as a component of the methylation hypothesis of epileptogenesis (Kobow and Blumcke, 2011). DNA methyltransferase enzymes 1 and 3a specifically, were increased in neurons from the temporal neocortices of 25 MTLE patients (Zhu et al., 2012). Using a rat model of MTLE, Williams-Karnesky et al. identified an increase in hippocampal DNA methylation that correlates with an increased DNA methyltransferase activity, disruption of adenosine homeostasis, and spontaneous recurrent seizures. To test the effects of adenosine, they used bioengineered silk implants to deliver a defined dose of adenosine over 10 days to the brains of epileptic rats (Williams-Karnesky et al., 2013). Adenosine implants reversed DNA hypermethylation seen in the epileptic brain, inhibited sprouting of mossy fibers in the hippocampus, and prevented the progression of epilepsy for at least 3 months (Williams-Karnesky et al., 2013).

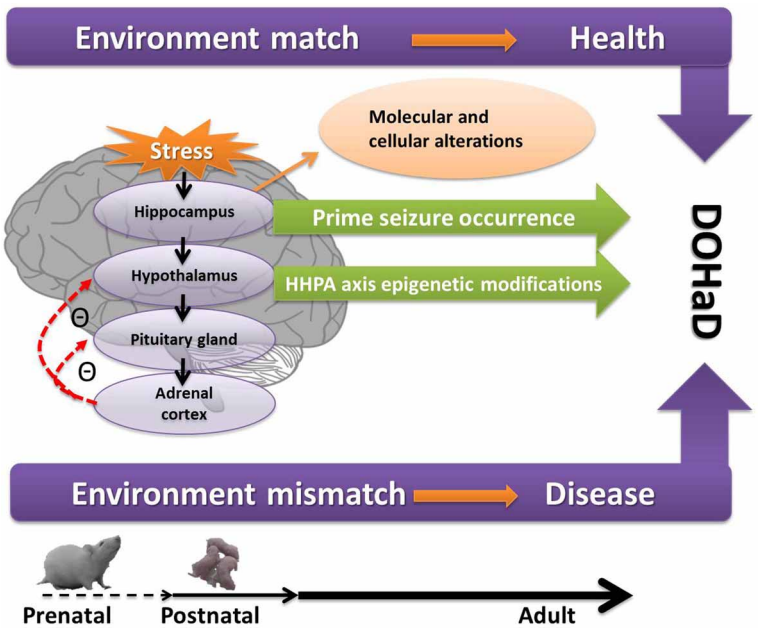


**FIGURE 1 | A conceptual diagram of how prenatal and postnatal stress act on the hippocampus and HPA axis and lead to neuropsychiatric disorders through epigenetic modifications, the so-called DOHaD as presently understood.** If the early programming environment matches the later adult environment, the adults are healthy. If mismatch occurs, the adults are more likely to have diseases. **Figure 1** also shows the increase seizure propensity in the context of early-life stress. See text for details. DOHaD, development origins of health and disease; HHPA, hippocampus and hypothalamic-pituitary-adrenal.

Thirdly, transcription factors are involved in epileptogenesis in human epilepsy patients. Repressor element-1 silencing transcription factor and neuronal restrictive silencer factor serve to repress gene expression through dynamic recruitment of epigenetic complexes (Qureshi and Mehler, 2009). Of interest, repressor element-1 silencing transcription has been implicated in the regulation of several epileptogenesis specific factors, including growth factors, neurotransmitter receptors, ion channels, circuit excitability, and neurogenesis (Huang et al., 1999; McClelland et al., 2011a,b; Roopra et al., 2012).

Fourthly, methyl-CpG-binding protein 2 can regulate neuronal activity and is itself controlled by activity (Roopra et al., 2012).

Taken together, early-life stress can prime seizure occurrence and increases epileptogenesis. In addition, epigenetic modification is a shared pathogenic substrate of early-life stress and epilepsy.

## COEXISTENCE OF EARLY-LIFE STRESS AND EARLY-LIFE SEIZURES

Seizure is one of the most common pediatric emergencies, with the highest incidence in the first year of life. Animal studies have demonstrated early-life seizures differ from adult seizures by the seizure behaviors, the electroencephalogram features, and their consequences. Notwithstanding the higher susceptibility to seizures, the immature brain is less vulnerable to seizure-induced injuries than the mature brain (Dube et al., 2001; Holmes and Ben-Ari, 2001; Huang et al., 2012). However, under some circumstances seizure in the immature brain can cause permanent brain damage (Dube et al., 2006).

For humans, most early-life seizures occur in premature and sick neonates (Scher et al., 1993; Miller et al., 2002; Scher, 2003) who are hospitalized and separated from their mothers, and thus, under stress (Field, 1994; Anand, 2000). Reciprocally, early-life stress may prime the occurrence of seizures and act via glucocorticoids, thereby potentiating the excitotoxic effects of concurrent neurological insults (Sapolsky, 1996), such as seizure (Huang et al., 2002a; Lai et al., 2006).

As stated above, early-life stress can prime the seizure occurrence and subsequent epileptogenesis. Currently, more attention is being paid to the effect of early-life stress on adult-onset seizure; however, little work has focused on the effect of early-life stress on the early-life seizure (Beck and Gavin, 1976; Edwards et al., 2002; Lai et al., 2006; Young et al., 2006; Velíšek, 2011; Yum et al., 2012). Indeed, to study the coexistence of early-life stress and early-life seizures is of both experimental and clinical importance.

## THE CONCEPT OF DEVELOPMENT ORIGINS OF HEALTH AND DISEASE (DOHaD)

Barker et al. noted that low birth weight was associated with an increased risk of adverse outcomes in adulthood, such as coronary heart disease, stroke, high blood pressure, and type 2 diabetes (Barker et al., 1989). Gluckman et al. proposed the concept of DOHaD by observing the enduring effects of the fetal environment on physical health and disease in adulthood. The process of fetal programming or developmental plasticity is one of the core assumptions of DOHaD (Gluckman et al., 2007). Gluckman et al. use the concept of predictive adaptive responses to describe the developing organism by making phenotypic responses during development to obtain an adaptive advantage (Gluckman et al., 2005). The fetus will predict and make adaptive responses to a broad range of environmental cues to aid fitness and survival in later life. If the prediction is correct, then there will be a good match between the phenotype adopted and the environment in which the organism will later live. If the prediction is poor, there will be a mismatch between the environment experienced and the phenotype induced. The authors propose that developmental mismatch triggers or exacerbates certain diseases and provide a useful explanation for the DOHaD phenomenon (Gluckman et al., 2005, 2007). Furthermore, the notion of epigenetic modifications is applied to the DOHaD approach (Waterland and Michels, 2007). The DOHaD approach has become so popular that an international society has been formed, and this society is actively promoting research and collaboration in this area.

**Figure 1** depicts the path form early-life adversity to long-term neuropsychiatric disorders, along with the underlying molecular and cellular mechanisms and epigenetic modifications, with a match or mismatch adaptation that leads to the final outcome.

## CONCLUSIONS AND PERSPECTIVES

Early-life stress can elicit detrimental effects on hippocampal development by altering the HPA axis, neuroplasticity, and behavior. Developmental plasticity allows an organism to adapt to environmental changes in the critical stages of early life. As highlighted in this review, early-life stress programs the development of the HPA axis, exerts profound effects on neural plasticity, primes seizure occurrence, and increases epileptogenesis. Epigenetic modifications play an important role in both early-life stress and epilepsy.

A number of important points made throughout the manuscript are reinforced here. Reducing damage done by prenatal and postnatal stress may help reduce the cost of treating adult diseases. Protecting pregnant mothers from harmful stress exposure and supporting programs to reduce stress or anxiety during pregnancy might lead to improvements in the health and well-being of their children later in life. Ideally, intervention and prevention should be achieved before pregnancy begins. In terms of postnatal stress, psychosocial interventions in early life can affect brain development and thereby benefit children at risk. Other perinatal adversities such as perinatal infection, nutritional disorders, and toxin exposures must be cautiously avoided and treated. The potential therapeutic value of pharmacological agents, such as $CRHR_1$ antagonists, MR and GR antagonists should be explored.

Recently, an increasing number of studies have shown that early-life stress primes seizure occurrence and increases epileptogenesis. An increased understanding of the link between early-life stress and epilepsy could improve the care and treatment of patients with epilepsy, while also allowing better management of other stress-related neurological disorders.

In the future, we will need to better determine the developmental windows during which preventative or therapeutic interventions can reverse the adverse effects of developmental programming. It will also be important to better understand

stress biomarkers, especially epigenetic biomarkers. An increasing number of studies have provided clues as to how early-life stress induces changes at the cellular, molecular, and epigenetic levels. Continued progress on these fronts will provide great insight into disease mechanisms, in turn leading to the potential identification of novel targets for therapy and prevention.

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# Early life stress and hippocampal neurogenesis in the neonate: sexual dimorphism, long term consequences and possible mediators

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Adverse early life experience decreases adult hippocampal neurogenesis and results in increased vulnerability to neuropsychiatric disorders. Despite that the effects of postnatal stress on neurogenesis have been widely studied in adult individuals, few efforts have been done to evaluate its immediate effects on the developing hippocampus. Moreover, it is not clear whether postnatal stress causes a differential impact in hippocampus development in male and female neonates that could be related to emotional deficits in adulthood. It has been proposed that the long term effects of early stress exposure rise from a persistent HPA axis activation during sensitive time windows; nevertheless the exact mechanisms and mediators remain unknown. Here, we summarize the immediate and late effects of early life stress on hippocampal neurogenesis in male and female rat pups, compare its later consequences in emotionality, and highlight some relevant mediator peptides that could be potentially involved in programming.

**Keywords: hippocampus, stress, neuroplasticity, stress mediators, gender differences**

## INTRODUCTION

Early life stress influences behavioral and physiological functions in the individuals, and results in long term changes in neuronal function increasing the vulnerability to suffer a psychiatric diseases (Felitti et al., 1998; Heim and Nemeroff, 2001). The hippocampus is involved in cognitive functions, and is an important regulator of emotional responses to stress, as it is one of the brain structures involved in glucocorticoid (GC) mediated HPA axis negative feedback. It is also one of the two niches where new neurons are actively produced throughout life. Early exposure to adverse experiences induces permanent changes in brain development that include alterations of the hippocampal formation and a reduced or altered neural plasticity. Since the hippocampus is especially vulnerable to GC induced toxicity, it has been hypothesized that stress exposure during sensitive time windows causes alterations on hippocampal development leading to a vicious circle, which perpetuates and exacerbates the long term consequences of early life stress (Sapolsky and Meaney, 1986). However, it is not totally clarified whether changes in neurogenesis originate as a result of many alterations in the hippocampal structure along the time line, or are an immediate consequence of stress exposure. Also, it is unclear whether neural plasticity is equally affected in male and female subjects at early ages. In the present work we will discuss the effects of early life stress on hippocampal developmental neurogenesis and will include a short summary of some possible mediators of stressful effects.

## THE STRESS RESPONSE

Stress involves the activation of the autonomic nervous and neuroendocrine systems to release a cascade of neurotransmitters, hormones, and other chemical messengers that induce behavioral and metabolic changes in the organism. A fast response is conveyed by the autonomic nervous system. A delayed response activates the hypothalamic paraventricular nucleus (PVN) to release corticotropin releasing hormone (CRH) to the portal vasculature of the anterior pituitary gland; CRH stimulates the release of adrenocorticotropin hormone (ACTH) which triggers the release of GCs from the adrenal cortex. GCs exert a negative feedback regulating HPA axis activity via its own receptors [glucocorticoid receptors (GRs) and mineralocorticoid receptors (MRs)] in anterior pituitary, hypothalamus (de Kloet et al., 2005a), hippocampus and prefrontal cortex. In adults, the effects of allostatic load dissipate following the removal of the stressor; however, the effects of early life stress are persistent far beyond the period of stress exposure.

## ANIMAL MODELS OF EARLY LIFE ADVERSITY

Animal models that reproduce many of the features of chronic stress or adverse experiences during early life include prenatal stress (PS) exposure (Lemaire et al., 2000), acute maternal deprivation (MD) procedures (de Kloet et al., 2005b), chronic or periodic maternal separation (MS) models (Sanchez et al., 2001; Huot et al., 2002; Plotsky et al., 2005), chronic early life stress (CES; Ivy et al., 2008), and early weaning of the pups (Kikusui and Mori, 2009). During late pregnancy, maternal GC mediate changes in fetal HPA responsiveness that is already functional. Infant rodents spend their first weeks of life in the maternal nest; hence, interactions of the pups with their mother and littermates are essential for optimal brain development and social skills (Sanchez et al., 2001; Huot et al., 2002). Separation from the dam for prolonged periods (>2 h) is perceived as a threat by the offspring, and activates

the neonate's HPA axis, causing elevated basal and/or stress-induced corticosterone levels in the adult (Lajud et al., 2012a). MD is an acute traumatic event that consists on separating the offspring from the dam for a 24 h period, and involves both nutritional and sensory (stimulation) factors (Suchecki et al., 1993), while MS is a chronic moderate stressor that involves daily separations from the dam during the first 2 or 3 weeks of life. It has been proposed that adult phenotype induced by MS is programmed by the pup's stressful experience during prolonged MS, rather than by prolonged maternal absence *per se* (Daskalakis et al., 2014). Variations in the MS model include daily separations of 3 h (MS180) or more (MS360), once or twice a day, from days 1–14, 2–21, or 15–21, etc. (Nishi et al., 2014). The CESs model, interferes with the mother infant interaction through the induction of a reduced maternal care, due to poor housing conditions (scarce material to build a nest) from PN2 to PN9, and resembles maternal anxiety and neglect (Ivy et al., 2008). These models reproduce many of the consequences observed in humans subjected to adverse early experiences, such as infant maltreatment or abuse, low socio economic status, etc., (Sanchez et al., 2001; Huot et al., 2002; Plotsky et al., 2005), in terms of a chronic exposure to adverse situations. Since effects of PS on neurogenesis have been more studied, we will review the studies on early neurogenesis focusing more on the effects of postnatal stress.

## DEVELOPMENT OF THE HIPPOCAMPUS

The development of the rodent dentate gyrus (DG) can be subdivided into two major phases. First, the granule cells of the outer shell (**Figure 1**, blue) originate prenatally from the neuroepithelium (NE) located near the fimbria and migrate from the progressively receding secondary dentate matrix to the subpial zone (SPZ; **Figure 1**, blue). The first dentate migration (dgml) is the source of the earliest generated granule cells that will constitute the outer shell of the granular layer (Altman and Bayer, 1990a,b; Li et al., 2009). During the second postnatal phase (**Figure 1**, red), the precursor cells build up a new proliferation zone distributed within the hilus, and the early embryonic radial glial scaffold from the ventricular zone (VZ) is replaced by a secondary glial scaffold that traverses the hilus (**Figure 1**, green). Most radial glial cells, support migrating neurons and serve as precursor cells for both neurogenesis and gliogenesis (Brunne et al., 2013). This tertiary dentate matrix peaks its proliferation rate between PND3 and PND10 and is responsible for the great increase in granule cell population during the neonatal period (Bayer, 1980). The granule cells (**Figure 1**, red) colonize either the outer shell or the inner core of the granule cell layer (GCL) in a symmetrical manner (Martin et al., 2002), and neurogenesis follows a characteristic dorso – ventral maturation gradient (Schlessinger et al., 1975). During the third and fourth weeks of life, the tertiary dentate matrix disappears and henceforth the neurogenic niche becomes

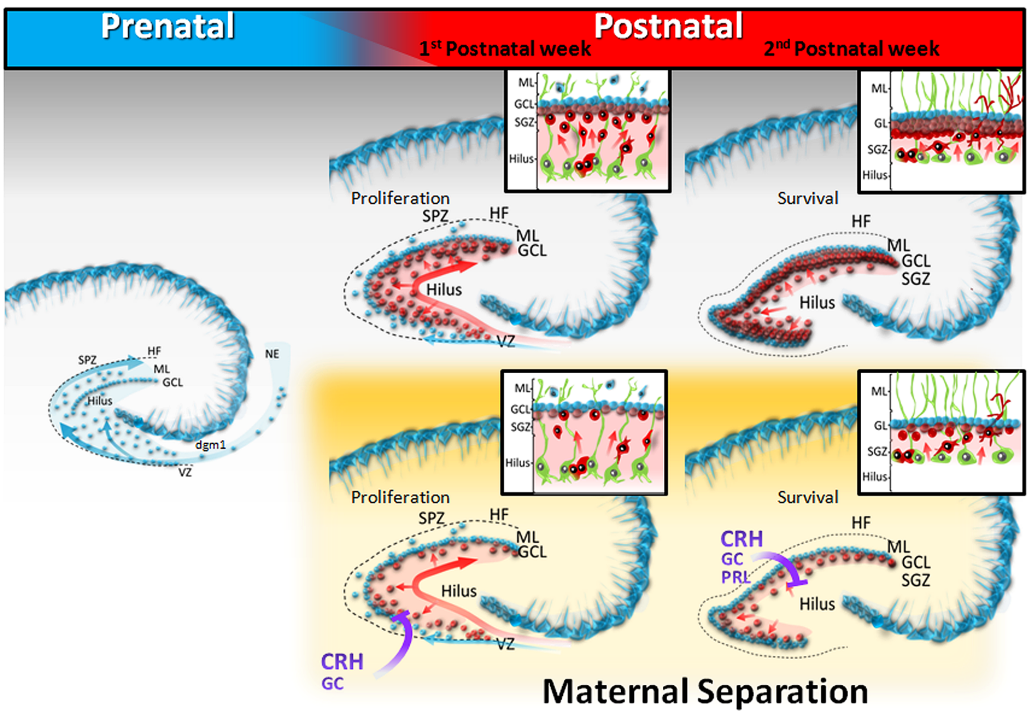


**FIGURE 1 | Schematic diagram of dentate gyrus development in postnatally stressed pups.** During prenatal development (E17-22), the granule cells of the outer shell (blue) originate from the neuroepithelium (NE), and migrate to the subpial zone (SPZ), or traverse the hilus. Throughout the first postnatal week, the precursor cells build up a new proliferation zone distributed within the hilus (light red), and granule cells of the GCL inner core migrate, following the arrangement of the secondary radial glial scaffold (green). During the second week of life, the neurogenic niche is confined to the subgranular zone (SGZ). Maternal separation decreases both proliferation and survival of new neurons, generated in the hilar tertiary dentate matrix, probably through stress – mediated mechanisms.

largely confined to the subgranular zone (SGZ; Altman and Bayer, 1990b). This SGZ is the main source of granule cells produced during early life and adulthood. For lifelong neurogenesis to occur, the DG must maintain the appropriate precursor cell niche in the SGZ, which is likely to be dependent on the developmental mechanisms at play during the DG formation. Stress exposure during the first weeks of life may have a significant impact on the maturation of the DG, as it disrupts the organization of the secondary and/or tertiary dentate matrix, altering permanently the structure and function of the hippocampus immediately after stress exposure.

## EARLY LIFE STRESS AND HIPPOCAMPAL NEUROGENESIS

Differences in neurogenesis between male and female pups have been recognized. More new BrdU+ cells were found in the DG of male rat pups compared to females at PN1 and PN4 (Zhang et al., 2008). Control males showed a higher proliferation rate, and increased survival of newborn cells, compared to control females. In addition, a larger granular cell layer volume and more young neurons (DCX) was found in males (Oomen et al., 2009). However, other group reported no differences on neurogenesis rates between male and female pups at PN15 (Lajud et al., 2013).

Early life adverse effects on adult hippocampal neurogenesis have been widely evaluated (Korosi et al., 2012), however the early effects during stress exposure period remain unclear. Immediate effects of stress exposure on hippocampal developmental neurogenesis, were initially addressed by Tanapat et al. (1998) who showed a reduced proliferation in the DG of male rat pups 24 h after a single stress exposure. MS also decreased the granular cell number in juvenile rats (Oreland et al., 2010). Early weaning in mice at PN15 induced fewer BrdU+ cells in the DG of male, but not female mice (Kikusui and Mori, 2009). MS (6 h/day) followed by early weaning (PN15) decreased cell proliferation in the DG of juvenile male rats (Baek et al., 2011, 2012). We showed that MS180 decreased the number and the density of BrdU+ cells in the DG of male pups, at PN15 (Lajud et al., 2012a). In contrast, increased cell proliferation and differentiation in the DG was found in male pups using the CES model (PND2 to PND9; Naninck et al., 2014). Since increased basal corticosterone levels were observed in CES pups (Naninck et al., 2014) but not after MS180 (Lajud et al., 2012a), we cannot exclude the possibility that the etiology of the adverse stimulus could exert differential effects. Maternally deprived pups (24 h PN3), showed decreased cell proliferation but not cell survival at PN21 (Oomen et al., 2009). In opposition to the studies in male pups, there are fewer reports concerning females. The number of BrdU+ cells was unchanged in the DG of female mice in response to early weaning (Kikusui and Mori, 2009). Naninck et al. (2014) showed an increased cell proliferation and differentiation in the DG of female CES pups. Preliminary studies from our group showed a decrease of cell survival in the DG of rat female pups at PN15, after MS180 (Lajud et al., 2012b). MD at PN3 found no changes in cell proliferation or survival in the DG of female pups at PN21, but only a decrease in the number of immature neurons (Oomen et al., 2009; **Table 1**).

In adults, SGZ neurogenesis has been studied with divergent results. For instance, Mirescu et al. (2004) reported that male adult rats subjected to MS180 exhibited decreased cell proliferation and survival with inadequate responses to stress; while Hulshof et al. (2011) observed that cell proliferation in the DG was decreased in adult MS180 rats but not cell survival. Other studies found that adult MS mice had similar rates of proliferating cells in the DG as control groups, but presented a lower survival rate and differentiation (Leslie et al., 2011). Additionally, male adult mice that were early weaned showed a reduced number of BrdU+ cells in the DG (Kikusui and Mori, 2009). In contrast, several studies found an increase in cell proliferation in adult animals, previously subjected to MS180 (Suri et al., 2013; Feng et al., 2014). In a subset of experiments, adult MS mice (8 h/day) exhibited an enhanced hippocampal neurogenesis in adulthood (Hays et al., 2012). Very few studies are done in adult females. Adult MD females showed reduced granule cell number and density in the DG (Oomen et al., 2010, 2011) or presented no effect. Despite a reduced neurogenesis before puberty (Loi et al., 2014) females subjected to limited nesting showed no effect in adulthood (Naninck et al., 2014). These results suggest that protective factors could take place in the female brain.

In summary, most of the studies show a trend of a decreased proliferation and/or a decreased cell survival in the DG of male and female rodents immediately after stress exposure, which could affect mainly the tertiary dentate matrix neurogenic niche. In adulthood, direction of changes is variable. In males, initial changes are sometimes followed by an increase, or by a permanent decrease in these parameters. In females, it seems that early effects of stress on neurogenesis subside in adulthood. In spite of the variety and direction of the changes that take place in the DG, it is assumed that early life stress induces such alterations to enable the individual to cope with future adversity in life (Bagot et al., 2009).

## LONG TERM CONSEQUENCES IN EMOTIONALITY

Early adversity has been linked to the development of psychiatric illness. A neurogenic hypothesis of depression was formulated, after findings of reduced hippocampal volumes in depressed patients, and the fact that chronic stress decreases hippocampal neurogenesis, and increases the risk to develop depression (Jun et al., 2012). Further, antidepressants were found to enhance hippocampal neurogenesis (Santarelli et al., 2003). However, the correlation of long term behavioral changes with hippocampal neurogenesis changes is still controversial due to several observations that neurogenesis and emotionality are independently regulated (Petrik et al., 2012). Proposals to reconcile the different results have been adressed (Eisch and Petrik, 2012).

Practically all the reports in animal models of long term consequences of early adversity in emotionality use adults. Studies vary, from no effects, to increases in anxiety and/or in depressive – like behavior in males (Newport et al., 2002; Daniels et al., 2004; Lee et al., 2007; Lajud et al., 2012a; Girardi et al., 2014; Nishi et al., 2014). In females, results are scarce but point to a lack of effect on contextual fear conditioning (Oomen et al., 2011), or anxiety (screened in the elevated plus maze; Grissom et al., 2012), but increase social anxiety (Tsuda and Ogawa, 2012). Fewer studies report changes in neurogenesis (**Table 1**) together with effects on

**Table 1 | Immediate effects of early life stress on neurogenesis, and long term effects on emotionality, in male and female rodents.**

| Authors | Early life stress procedure | Species | Sex | Age | Effects on hippocampus | Emotional behavior in adulthood |
|---|---|---|---|---|---|---|
| Tanapat et al. (1998) | Acute exposure to the odor of a predator on PN6 | Rats | Males | PN7 | Decreased cell proliferation in the DG. | |
| King et al. (2004) | Prenatally malnourished (PMN) pups | Rats | Males | PN7, PN30 | Reduced cell proliferation in the fascia dentata at PN7, but significantly higher on PN30. | |
| Oomen et al. (2009) | 24 hMD on PN3 | Rats | Males (M) females (F) | PN4, PN21 | SEX DIFFERENCES found between control males and females.<br>PN4: no changes in cell proliferation.<br>PN21: (M) decreased cell proliferation, no effect on cell survival, increase in cell differentiation. (F) no effect on cell proliferation or cell survival, reduced cell differentiation. | |
| Raceková et al. (2009) | MS180 from PN1-PN14, or PN1 – PN21 | Rats | Males | PN14, PN21, PN28 | MS decreased proliferation in the Rostral Migratory Stream in all experimental groups. | |
| Oreland et al. (2010) | MS15 or MS360 from PN121 | Rats | Males | PN22 | MS360 decreased number of neurons and cell density in the DG, compared to MS15. | |
| Baek et al. (2011) | MS360, (PN1-14), and early weaning at PN15 | Rat | Males | PN28 | Decreased cell survival and cell differentiation.<br>Reduced cell density in the DG. | |
| Lajud et al. (2012a) | MS180 from PN2 – 14 | Rat | Males | PN15 | MS180 decreased cell survival and cell differentiation.<br>Reduced cell density in the DG. | Increased depressive – like behavior; no effect on anxiety. |
| Lajud et al. (2012b) | MS 180, PN2 – 14 | Rats | Females | PN15 | Decreased cell survival in the DG. | |
| Baek et al. (2012) | Early weaning on PN14 and later isolation | Rats | Males | PN35 | Decreased cell proliferation in the DG. | |
| Wang and Gondré-Lewis (2013) | MS180 from PN2-21 with or without prenatal nicotine exposure | Rats | Males and females | PN14 | MS180 increased pyramidal neurons in CA1. In the DG, MS180 decreased number of granule neurons. | |
| Lajud et al. (2013) | Prolactin, vehicle, or left undisturbed from PN1 – 14 | Rats | Males, females | PN15 | No differences in cell number or cell density between control males and females. Prolactin decreased cell survival in the DG. | |
| Naninck et al. (2014) | ES Limited nesting PN2 – 9 | Mice | Males (M) females (F) | PN9, adulthood | PN9: increased cell proliferation and differentiation and number of immature cells in the DG. Adulthood: (M) reduced cell survival of adult born neurons. (F) No differences observed in adult females. | No effect on anxiety in the EPM, similar depressive- like behavior as controls. |

*(Continued)*

**Table 1 | Continued**

| Authors | Early life stress procedure | Species | Sex | Age | Effects on hippocampus | Emotional behavior in adulthood |
|---|---|---|---|---|---|---|
| Loi et al. (2014) | Comparison of prenatal, and postnatal stress (24 h MD) | Rats | Males (M) females (F) | Juveniles, adulthood | 24 h MD enhances neurogenesis until the onset of puberty in male rats. Reduced neurogenesis in females prior puberty. The effect subsides in adulthood. | |
| Huot et al. (2002) | MS180 or MS15, on PN2 – 14 | Rats | Males | Adulthood | Decreased mossy fiber density in the stratum oriens region but no change in volume of the DG. | |
| Mirescu et al. (2004) | MS 180, PN2 – 14 | Rats | Males | Adulthood | Decreased cell proliferation and decreased immature neuron production in the DG. | |
| Fabricius et al. (2008) | 24 h MD on PN9 | Mice | Males and females together | Adulthood | Decreased cell number and cell proliferation in the DG in MD mice. | Reduced anxiety in the elevated plus maze. |
| Aisa et al. (2009) | MS180 PN2 – 21 | Rats | Males | Adulthood | Reduced cell proliferation. Synaptic plasticity markers were decreased (NCAM, and synaptophisin, BDNF mRNAs). | |
| Karakaş et al. (2009) | 24 h MD on PN4, PN9, or PN18 | Rat | Males (M) females (F) | Adulthood | MD produced decreased the number of synapses in CA1 and CA3 subregions of the hippocampus. No differences between M and F were found. | |
| Kikusui and Mori (2009) | Early weaning in mice at PND15 | Mice | Males (M) females (F) | Adulthood | M showed decreased BDNF levels in the hippocampus and prefrontal cortex, and reduced cell survival in theDG. No effect on F. | |
| Bagot et al. (2009) | Maternal care (high or low licking and grooming) | Rats | Males | Adulthood | Less dendritic complexity in low LG offspring compared to high LG. | |
| Oomen et al. (2010) | 24 hMD on PN3 | Rats | Males | Adulthood | MD decreased cell proliferation, cell survival, and neuronal differentiation. MD reduced total cell number in the caudal part of DG. | MD impaired spatial learning but enhanced contextual fear conditioning. |
| Leslie et al. (2011) | Unpredictable MS180 from PN1 – 14 | Mice | Males and females together | Adulthood | No change in cell proliferation in the DG, but reduced cell survival and differentiation into neurons. Plus, less complex dendritic arborization and fewer dendritic spines. | |
| Oomen et al. (2011) | 24 h MD on PN3 | Rats | Females | Adulthood | No significant differences between groups in proliferation, cell survival or neuronal differentiation. MD reduced total cell number and cell density in the GCL. | No differences in spatial learning and contextual fear conditioning . MD improved amygdala-dependent fear memory. |

*(Continued)*

**Table 1 | Continued**

| Authors | Early life stress procedure | Species | Sex | Age | Effects on hippocampus | Emotional behavior in adulthood |
|---|---|---|---|---|---|---|
| Hulshof et al. (2011) | MS180 from PN1 – 14. | rats | males | adulthood | Cell proliferation was decreased in the DG of MS animals. Cell differentiation and survival were not altered. | No changes in anxiety-like behavior, explorative behavior and social interaction, but affected cognitive function (object recognition task). |
| Hays et al. (2012) | Morphine injections or exposure to MS 480 | Mice | Males | Adulthood | Both neonatal stress or morphine treatment increased hippocampal neurogenesis in adult mice. | |
| Korosi et al. (2012) | Review | | Males, females | Different ages | | |
| Suri et al. (2013) | MS | Rats | Males | Adulthood | Enhanced hippocampal neurogenesis, with enhanced BDNF levels. | |
| Feng et al. (2014) | MS | Rats | Males | Adulthood | Increased cell proliferation in the subventricular zone, cortical layer 1 and hippocampal dentate gyrus. | |

*We show a summary of the studies addressing the effects of early life adversity on different parameters of developmental neurogenesis, indicating sex, species, type of stress exposure, and age of the animals. Effects on neurogenesis in adult animals are also indicated, as well as long term effects on emotional behavior, where applicable.*

emotionality (Fabricius et al., 2008; Hulshof et al., 2011; Oomen et al., 2011; Lajud et al., 2012a; Naninck et al., 2014). Thus, it seems that in rodents, males are more vulnerable to the effects of early adversity than females.

## EARLY LIFE STRESS EFFECTS ON DG DEVELOPMENT AND SOME POSSIBLE MEDIATORS

We highlight two well-known factors mediating the stress response, and two peptide messengers as potential mediators.

## GLUCOCORTICOIDS

During the first 2 weeks of life, rat pups experience a stress hyporesponsive period (SHRP) due to a markedly reduced adrenocortical response to stress (Sapolsky and Meaney, 1986). GC administration during the first postnatal week decreases granule cell survival, and results in a significant increase in the density of both cell proliferation and death, within the hilus neurogenic niche (Gould et al., 1991). Since then, increased circulating GC levels have been proposed as the main mediators of early life stress effects on hippocampus developmental neurogenesis (Gould and Tanapat, 1999). GCR gene expression is present within the developing brain since early fetal development (Yi and Baram, 1994), and is maximally expressed in the DG between PND10 and 16 (Yi and Baram, 1994); however, other mediators could be involved in the psychopathology of early life stress (Schmidt et al., 2009; Lajud et al., 2012a; Horii-Hayashi et al., 2013; Liao et al., 2014). GCR regulate CRH gene promoter in the mature brain. In the neonate, second messenger cascades are not yet functional, and GC levels fail to modify CRH expression. Thus, GCR may mediate different functions in the developing neurons, possibly mediated by trophic effects (Yi and Baram, 1994).

## CORTICOTROPIN RELEASING HORMONE

The CRH is considered the link between early life adversity and adult vulnerability (Brunson et al., 2003). CES and MS permanently increase CRH expression within the hippocampus (Ivy et al., 2010; Wang et al., 2014). Administration of CRH into the brains of infant rats recapitulates some of the long term effects associated with early life stress, even when GC levels are clamped at physiological levels (Brunson et al., 2001; Wang et al., 2012). Blockade of CRHR1 signaling during adulthood significantly attenuated the hippocampal synaptic dysfunction, and memory defects in maternally separated rats (Wang et al., 2014), and treatment blocking CRHR1 from PN10-17 prevented ELS – induced augmentation of hippocampal in middle-aged rats (Ivy et al., 2010). Notably CRHR1, but not GCR, antagonism during the developmental critical period attenuated ES – induced endocrine alterations (Ivy et al., 2010; Liao et al., 2014). Moreover, a specific population of Cajal-Retzius-like CRH-expressing neurons was characterized during early postnatal hippocampus and these cells seem to contribute to the establishment of hippocampal connectivity (Chen et al., 2001).

## PROLACTIN

Prolactin (PRL) is a pleiotropic hormone promoting a vast array of effects (Freeman et al., 2000). PRL is released in response to stress, regulates hippocampal and SVZ neurogenesis, and modulates anxiety and HPA axis reactivity (Torner et al., 2001, 2009; Shingo et al., 2003). PRL enters the brain through its receptors, located in the choroid plexus cells (Walsh et al., 1987). Daily PRL administration (PN1 to PN14) induced a decrease of DG neurogenesis of PN15 pups (Lajud et al., 2013), and increased depressive – like behavior, in adult male and female rats (Lajud et al., 2013). Studies showed that PRL enhances CRH (Blume et al., 2009) and AVP expression (Donner and Neumann, 2009; Vega et al., 2010). Additionally PRL is cleaved to produce vasoinhibin, which has opposite actions of the native hormone (Zamorano et al., 2014). Thus, PRL could contribute to stress programming.

## CITOKINES

Prenatal maternal infections increase the risk of developing schizophrenia or autism in the offspring (Kofman, 2002; Musaelyan et al., 2014). Immune activation during the perinatal period increases cytokine production, particularly of Interleukin 1 beta (IL-1b) and tumor necrosis factor-α, in the hippocampus (Diz-Chaves et al., 2012). Treatment of hippocampal neurospheres with IL-1b showed an antiproliferative, antineurogenic and progliogenic effects (Green et al., 2012). Further, IL-1b reduced the serotonergic differentiation of cultured neurospheres in a dose-dependent manner (Zhang et al., 2013). Several studies reported either increases or decreases in cell proliferation or cell survival in the hippocampus offspring depending on the time of exposure and the time of neurogenesis assessment (Musaelyan et al., 2014). Further, mice pups given an immune challenge at PN9, showed reduced cell proliferation, and reduced cell number of neural progenitors at PN41 (Järlestedt et al., 2013). Thus, cytokines play an important role during brain development.

## CONCLUDING REMARKS

Early adversity disrupts the normal concentrations of important neurotransmitters, peptides, hormones, cytokines or their receptors, which are either expressed in the brain or enter the brain compartment during development. These alterations influence the local milieu in the DG, possibly affecting the tertiary dentate matrix and producing a decrease of granular neurons, a decreased cell survival and/or differentiation (**Figure 1**). Compensatory mechanisms, such as differential expression of neurotrophic factors, might, or not, induce a secondary increase of granular neuron synthesis at adult ages, in both male and female rodents. In any case, the alteration of the development of the structure and compensatory mechanisms induce permanent changes in hippocampal function, which are sometimes accompanied by increased anxiety or depressive-like behavior in males.

## AUTHOR CONTRIBUTIONS

Author Naima Lajud, managed the literature searches, made substantial contributions to the conception or design of the work; wrote a part of the manuscript and revised the work critically, and designed **Figure 1**. Author Luz Torner made the conception and design of the work, managed the literature searches, made **Table 1**, wrote a part of the manuscript, revised the work critically, and wrote the final draft. Both authors contributed to and have approved the final manuscript.

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REVIEW
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# Gene-environment interaction in programming hippocampal plasticity: focus on adult neurogenesis

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Interactions between genes and environment are a critical feature of development and both contribute to shape individuality. They are at the core of vulnerability resiliency for mental illnesses. During the early postnatal period, several brain structures involved in cognitive and emotional processing, such as the hippocampus, still develop and it is likely that interferences with this neuronal development, which is genetically determined, might lead to long-lasting structural and functional consequences and increase the risk of developing psychopathology. One particular target is adult neurogenesis, which is involved in the regulation of cognitive and emotional processes. Insights into the dynamic interplay between genes and environmental factors in setting up individual rates of neurogenesis have come from laboratory studies exploring experience-dependent changes in adult neurogenesis as a function of individual's genetic makeup. These studies have implications for our understanding of the mechanisms regulating adult neurogenesis, which could constitute a link between environmental challenges and psychopathology.

**Keywords: genetics, environment, life events, hippocampus, neurogenesis, strain**

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## Introduction

Clinical studies related both to neurodegenerative and psychiatric illnesses indicate that gene-environment interactions play an important part in the expression or the etiology of these diseases. Thus, examples of genetic pathologies that are differently expressed according to the environment (Migliore and Coppedè, 2009), such as huntington disease (Mo et al., 2015), Alzheimer disease (Swaminathan and Jicha, 2014) or Parkinson disease (Kieburtz and Wunderle, 2013) are now available, and both epidemiologic and clinical reports point to an important role of life events in precipitating mental disease (Caspi et al., 2003; Caspi and Moffitt, 2006).

In this context, because they act during critical developmental periods, environmental factors at play during early life have definitive "reprogramming" effects and gene-early life environmental factors were shown to interact to define vulnerability or resiliency for mental illnesses in adulthood (Heim et al., 2010). During the early postnatal period, several brain structures involved in cognitive and emotional processing, such as the hippocampus, still develop (Schlessinger et al., 1975; Altman and Bayer, 1990). It is thus very likely that interferences with this neuronal development, which is genetically determined, might lead to long-lasting structural and functional consequences and increase the risk of developing psychopathology. A particular feature of the dentate gyrus (DG) is the ability to produce new neurons in adulthood, a process that was shown to contribute to specialized functions such as

learning and memory (Koehl and Abrous, 2011) and control of anxiety and mood states (Revest et al., 2009), hence the interest in the different factors involved in its regulation.

Although a central issue in psychiatry is to determine how the gene-environment interactions can explain individual differences in vulnerability/resiliency to psychopathology, in the context of adult neurogenesis, genetic and environmental factors are often studied alone in approaches that attempt to reduce the experimental variation by focusing on one strain. Nevertheless a few examples of the literature have tackled the importance of this interaction, which will be the focus of this review after a brief presentation of methods in gene-environment studies and of adult neurogenesis.

## Importance of Gene-Environment Interplay in Shaping Phenotype: A Historical Overview

Our current scientific opinion regarding the origin of individual differences in personality, aptitudes, and behavioral traits in general, is that neither genetic nor environmental differences are solely responsible for producing phenotypic variation, and that virtually all traits result from the joint influence of genetic and environmental factors. However, this consensual view took time and effort to prevail, and heated nature vs. nurture debates, which assumed that variation in a trait is primarily due to either genetic or environmental differences, were opposing scientists in the 1940's and 1950's.

The original compromise and recognition that both genes and environmental factors could explain a part of the phenotypic variance led to a simple equation: Phenotypic variance ($V_P$) = Genetic variance ($V_G$) + Environmental variance ($V_E$), and two major approaches have been developed and used by behavior geneticist to analyze the respective contribution of both factors: the first one consists in studying the effects of environment alone by holding the genetic make-up of the individual constant ($V_P = V_E$). In essence this consists in placing individuals of the same genotype in different environments and comparing their phenotypic response. This approach, which can be easily developed in humans—comparing twins raised in different families (Bouchard et al., 1990)—, benefits from the existence of inbred strains of mice in which all individuals are isogenic, allowing to directly test the influence of environmental impact. As one can imagine, the second approach consists in studying the effects of genes alone by holding environment constant ($V_P = V_G$). Typical example is the use of knock-outs (KO), mainly in mice, or selective breeding of rats for a specific trait that is selected and enriched across generations. One of the first influential studies addressing this aspect is a classic selective breeding study in which Tryon (1942) selected rats for their ability to find their way in a maze. He mated the animals that made the fewest errors (maze bright) together and the ones that made the most errors (maze dull) together. Then he mated the most similar offspring for 21 generations, and after seven generations, he had already developed two genetically different lines of rats (maze bright and maze dull). This study was highly influential in the field of psychology for showing that specific behavioral traits may be hereditary.

Finally, with the recognition that phenotypic variance was sometimes not explained by the simple additive contribution of genetic and environmental factors, a third term $V_{GE}$, which measures how much of this variance is due to an interaction between the genotype and the environment, has been introduced. It can be experimentally approached by comparing the effects of different environments on different genotypes. Among the first examples of this approach, Cooper and Zubek compared the performances of Tyron's rats when raised in either a restricted (an empty cage with gray walls) or an enriched environment (EE; a cage with designs on its walls that contained objects such as ramps, mirrors, swings, balls, slides, and tunnels). They observed a drastic decrease in the performances of bright rats when raised in the restricted environment, which did not affect the already bad performances of dull rats, and an improvement of the performances of dull rats that reached bright rats levels when raised in an EE. With regard to these results, Cooper and Zubek argued that heredity and environment always interact to produce final behavior (Cooper and Zubek, 1958).

Following up this seminal experiment, many studies have been conducted along that line, among which I selected two as they have been, to my point of view, highly influential in the field. In the first one, Crabbe et al. (1999) compared the behavior of mice from different strains in different labs, but using the exact same protocols and apparatus. He showed as expected that genotype was a highly significant parameter for all behaviors studied, accounting for 30–80% of the total variability, and that several documented strain differences were confirmed. However he also found that despite standardization, there were systematic differences across labs, and that for some tests, the magnitude of genetic differences depended upon the testing lab. Altogether the authors highlighted that given the importance of the gene-environment interactions revealed in their study, "***experiments characterizing mutants may yield results that are idiosyncratic to a particular laboratory***". The second study performed by Francis and colleagues aimed at distinguishing genetic and early environment contributions to the expression of adult behavior in mice. To this end, they used classical fostering approaches, and investigated the effects of prenatal (embryo transfer) and postnatal (cross-fostering) environments in two strains of inbred mice with profound and reliable differences in behavior, namely C57Bl/6J and Balb/cJ mice. They found that C57Bl/6J mice that developed in a Balb/cJ uterus and were reared by a Balb/cJ mother displayed a Balb/cJ phenotype in most behavior tested (exploration, anxiety, spatial learning), while C57Bl/6J mice exposed only to a uterine or a postanatal Balb/cJ environment did not change their phenotype. For the first time, this dataset emphasized the crucial interaction of pre- and post-natal environments in shaping the development of selected behaviors, and further indicated that prenatal environment may prime the developing pup to respond to postnatal environment—most certainly cues delivered by maternal care—in a way that would allow a strain-specific behavior to develop independently of genotype (Francis et al., 2003). Although this study very elegantly demonstrated

the importance of gene-environment interactions, one can regret that the same experimental design was not applied to Balb/cJ mice as it is highly probable that these environmental influences can act only within a genetic constraint and that different sensitivities can be expected depending on genetic background, a hypothesis that remains to be tested as of today.

## The Biology of Adult Hippocampal Neurogenesis

Neurogenesis was until quite recently thought to be specifically an ontogenetic aspect of Central Nervous System (CNS) development. However during the last 20 years there has been unequivocal evidence that new neurons are produced in adulthood, not only in lower vertebrates, but also in mammalian species including humans. Two discrete zones of the adult brain have been described as neurogenic: the olfactory bulb and the DG of the hippocampal formation, the focus of this review.

Adult neurogenesis is permitted by the maintenance in the DG of a neurogenic niche that derives from the tertiary dentate matrix mostly active during the early postnatal period and from which most granule cells of the DG are originating (for review, see Lajud and Torner, 2015). This neurogenic niche is composed of neural stem cells and progenitors that produce immature new neurons; these migrate locally into the granule cell layer (GCL) to become dentate granule cells. Adult neurogenesis appears to recapitulate the complete process of neuronal development, ranging from neural progenitor activation and fate determination, to differentiation, migration, and axonal and dendritic development of newborn neurons, to synapse formation and functional integration into the existing neural circuitry (**Figure 1**).

Adult neurogenesis is tightly regulated by the local environment, the so called "neurogenic niche" that is composed of the extracellular matrix and various cell types, including astroglia, ependymal cells, endothelial cells, immature progeny of adult neural stem cells and mature neurons within the local circuitry. The niche is also a target of many physiological, pathological and pharmacological stimuli that regulate adult neurogenesis.

## Gene-Environment Interplay in Controlling Adult Hippocampal Neurogenesis

Both genetic and environmental contributions to adult neurogenesis have been, and are still, widely studied. Thus in the course of the last 20 years, direct strain comparisons and analysis of the genetic contribution to the variance observed in adult neurogenesis has shown that genetic variation among strains accounted for differences in all aspects of hippocampal neurogenesis, proliferation, survival and differentiation, as well as overall hippocampal volume and total cell numbers (Kempermann et al., 1997a), and that 85% of the variance in neurogenesis between strains could be accounted for by different cell-survival rates while proliferation was only a mild predictor of neurogenesis (Kempermann et al., 2006). Many genes have been singled out that control the different steps of neurogenesis, constituting a molecular signature (Miller et al., 2013), and we refer readers to the MANGO database that lists the different genes involved in controlling non pathological neurogenesis (Overall et al., 2012). In regard to these data, the prevailing view is that adult neurogenesis is a complex phenomenon that is likely to be controlled by the interaction of several regulatory loci involving many genes, and not one master regulatory locus acting as a switch to turn neurogenesis "on" or "off". In parallel, many studies have shown that different life events were capable of regulating or controlling neurogenesis, and both adult experience (Opendak and Gould, 2015) and early life events (for review, see Fenoglio et al., 2006; Korosi et al., 2012; Lucassen et al., 2013; Hoeijmakers et al., 2014) largely contribute to its levels, the impact of early life events appearing more pervasive and permanent, certainly for they act during developmental periods.

On the opposite, studies carefully controlling both genetic and environmental factors in order to assess the importance of their interactions in determining adult neurogenesis are scarce. The most important ones are those related to the impact of early life environment for the known role of factors acting during this period in shaping phenotypes, and I will focus on these studies. However, because there are only a few of them, and because much information can be gained from the analysis of gene-later life environment interactions, I will also detail these latter studies.

### Impact of Early Life Environment

Many studies have reported that prenatal stress (PS) plays a very influential role in determining the rate of adult neurogenesis (Lemaire et al., 2000; Ortega-Martínez, 2015), but very few have visited this question in relation to genetic background. To the best of our knowledge, only one study by Lucassen and colleagues addressed this issue and compared the impact of PS on early postnatal neurogenesis between rats genetically divergent as issued from a selective breeding for high- and low-anxiety-related behavior, high anxiety bred (HAB) and low anxiety bred (LAB) respectively (Lucassen et al., 2009). As hypothesized, the effects of PS were found to be dependent on the genetic background of the mother and the survival rate of newborn cells and the number of immature neurons doublecortin immunoreactive (DCX-IR) were significantly altered in offspring of stressed HAB rats compared to control HAB but not in offspring of LAB rats. Authors also tested whether the different sensitivity of HAB and LAB rats may be related to placental 11β-HSD2 levels as their variations were found to represent a physiological link between environmental challenges to the pregnant female and programming of the fetal brain, and found increased levels of 11β-HSD2 activity in PS-LAB, suggesting it may have a protecting effect against the programming consequences of PS.

The impact of the postnatal period has been addressed in mice, a gold standard model for this type of analysis because they offer a large variety of genetic backgrounds, but again these studies are scarce. Thus neurogenesis was found to be insensitive to maternal separation (Navailles et al., 2008) in mice from two strains known to differ in their stress reactivity

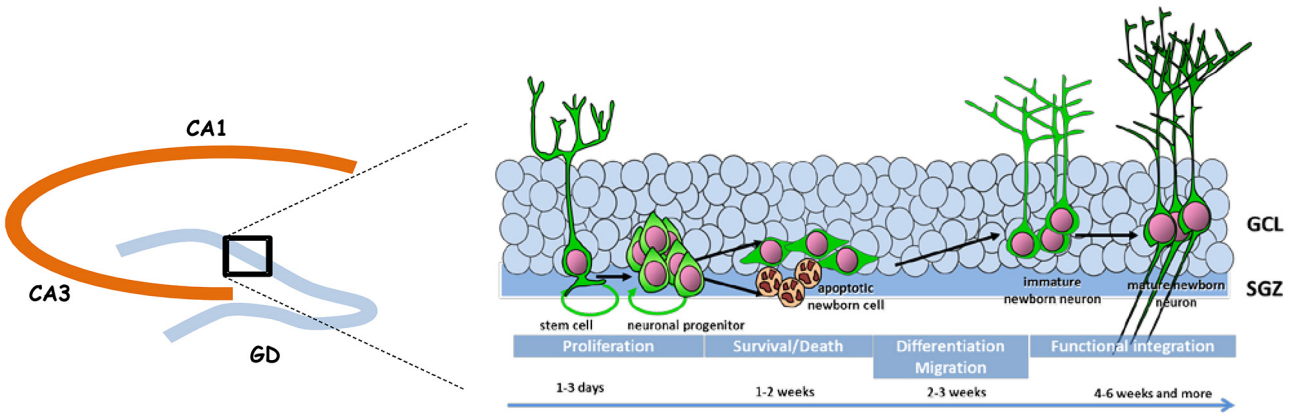


**FIGURE 1 | Schematic representation of adult neurogenesis.** New neurons that integrate the granule cell layer (GCL) of the dentate gyrus (DG) originate from stem and precursor cells located in the subgranular zone (SGZ). In the course of their maturation they exhibit specific properties that confers them a unique behavioral function.

and their levels of neurogenesis: C57Bl/6J, which exhibit a strong resistance to stress—albeit not to early life events (Francis et al., 2003)—, and high levels of neurogenesis, and Balb/cJ, which are known for their liability to stress and low levels of neurogenesis (Kempermann et al., 1997a). Interestingly, this postnatal manipulation strongly inhibits neurogenesis in outbred rats (Mirescu et al., 2004; Oomen et al., 2010), thus raising the question of strain sensitivity to postnatal developmental forces. In order to address this question, we have developed a model allowing to single out the influence of the genetic make-up of the individual on the outcome of maternal care on adult neurogenesis. We selected C57Bl/6J mice and DBA/2J mice for their differences in baseline neurogenesis (Kempermann and Gage, 2002) as well as in their sensitivity to environmental experiences, DBA/2J mice appearing more vulnerable than C57Bl/6J mice (Cabib et al., 2000). Mice from both strains were raised by mothers of non-related strains that displayed high and low levels of maternal care, independently of the strain of fostered pups. This experimental design allowed us to compare neurogenesis in mice from two different genetic backgrounds exposed to the same environmental influences. We reported that maternal care had a major impact on neurogenesis—targeting both the number of immature newborn cells and their morphology—exclusively in DBA/2J mice, thus genetically prone to respond to these influences (Koehl et al., 2012). Interestingly, we had previously reported that DBA/2J mice raised in a high maternal care environment exhibited an anhedonic endophenotype (van der Veen et al., 2008) that could be linked to the delayed maturation of immature granule cells, while C57Bl/6J mice showed resiliency to both the neuroanatomical and behavioral consequences of variations in maternal care. This is particularly relevant to the human condition as most psychiatric theories relate the development of depression to a disruption of mother-infant interactions in certain vulnerable individuals (Rutter et al., 2006), and as neurogenesis has been linked to the effects of antidepressants (see below).

Finally, taken together with results of Navailles et al. (2008), this study confirms that the setpoint for adult neurogenesis in C57Bl/6J mice appears independent of the postnatal environment and a rapid analysis would lead to the conclusion that this setpoint is determined prenatally for C57Bl/6J mice and postnatally for DBA/2J mice, while it may not be influenced by early environment in Balb/cJ mice. However, because an interaction of both pre and postnatal environments was found to be necessary to influence behavior in adult C57bl/6J mice (Francis et al., 2003), and because some postnatal manipulations, such as handling, have no net impact on neurogenesis but can counteract the impact of PS in outbred rats (Lemaire et al., 2006), we cannot exclude that neurogenesis in C57Bl/6J and Balb/cJ mice is determined by a combination of pre and postnatal factors that need to play in synergy.

## Impact of Enriched Environment and Voluntary Exercise

Early life events are not the only shaping factors of adult neurogenesis that have been studied in the context of gene-environment and researchers have showed a lot of interest for the impact of voluntary exercise, one of the most potent adult regulator of neurogenesis. The first evidences of genetic differences in response to complex environment in adulthood are indirect and emerge from comparing results from different studies. Thus in his seminal paper published in 1997 in Nature, Gerd Kempermann reported that upbringing weanling female mice from the C57Bl/6J strain in an EE for 40 days elicited a robust increase in the survival of newly born cells while it had little or no influence on their proliferative activity (Kempermann et al., 1997b). In parallel, he also reported the existence of a drastic variation in baseline levels of precursor proliferation among different mouse strains (Kempermann et al., 1997a), with mice from the 129/SvJ strain having extremely low levels of adult neurogenesis compared to most other inbred strains, and in particular C57Bl/6Jmice. Using the exact same environmental procedure as in his earlier study, he then analyzed

the impact of EE in these mice, and reported that in contrast to C57Bl/6J mice, it increased proliferation of progenitor cells as well as the net number of surviving cells, although it actually decreased their survival rate, in 129/SvJ mice. Although he did not run a direct comparison of the two strains responsiveness, he found that the net neurogenic effect of EE was similar in C57Bl/6J and 129Sv/J mice, but that the mechanisms involved differed. This is consistent with the fact that proliferation, survival and differentiation of progenitor cells and their progeny are each separately influenced by inheritable traits and not uniformly upregulated in response to environmental stimulation (Kempermann et al., 1998).

Following up on this seminal discovery, Van Praag and colleagues attempted to separate components of the EE that could explain its pro-survival effect and focused on physical activity (van Praag et al., 1999). They showed in the same mouse line (C57Bl/6J) that voluntary exercise in a running wheel strongly increased cell survival, albeit to a lower extent than EE. It also strongly stimulated cell proliferation, an effect not observed in the EE condition in this strain. Since then the sensitivity of divergent inbred strains of mice to voluntary exercise has been complemented by direct strain comparisons. Thus a study comparing the neurogenic response to exercise in 12 isogenic mouse strains reported that although exercise increased neurogenesis in all 12 strains tested, the magnitude of the effect depended on genotype (Clark et al., 2011). In particular, C57Bl/6J mice, which are the most widely used in studies related to exercise that do not take into consideration genetic influences, was found to be the least responsive strain. Furthermore, a significant percentage of the strain variation in exercise-induced neurogenesis could be accounted for by the distance the animals ran, but removing this factor did not abolish the strain effect, indicating that the quantitative increase in total number of new neurons resulting from exercise differs between strains with some strains showing relatively more new neurons for the same amount of running as compared to others. For example, 129-related strains were found to display a very strong relationship between level of wheel running and number of new neurons, C57Bl/6J mice were intermediate, and DBA/2J showed near zero or negative correlations, indicating no relationship between wheel running and number of new neurons (Merritt and Rhodes, 2015). The same type of observation was reported in another study specifically comparing the effects of exercise on C57Bl/6J and DBA/2J mice, which even reported that although running has pro-proliferative and pro-survival consequences in C57Bl/6J mice, it has only a delayed pro-proliferative effect in DBA/2Jmice that is not correlated to the amount of running (Overall et al., 2013). Altogether this discrepancy between the two strains comforts indications that proliferation and survival programs are mediated by different mechanisms (Kempermann et al., 2006). Interestingly it was also reported that genetic variation in neurogenesis under standard housing conditions was unrelated to running levels of neurogenesis, suggesting that different genes influence variation in adult hippocampal neurogenesis under sedentary vs. runner conditions. Authors further analyzed heritability (the proportion of differences of a trait among individuals of a population that are due to genetic differences), and reported a heritability score of 0.53 for sedentary and 0.33 for runner mice, all strains confounded (Clark et al., 2011).

## Impact of Pharmacological Treatment: Example of Fluoxetine

Similarly to the impact of EE and voluntary exercise, first evidences of strain differences in neurogenic sensitivity to pharmacological treatments are indirect. Thus after pro-proliferative consequences of treatments with serotonin-specific reuptake inhibitors (SSRI) antidepressants such as fluoxetine were reported (Malberg et al., 2000), Santarelli and colleagues further analyzed the neurogenic consequences of this treatment and reported an increase in cell proliferation (Santarelli et al., 2003) as well as a stimulation of dendritic maturation of newborn cells (Wang et al., 2008) in 129SvEv mice. They also showed that disrupting the neurogenic effects of fluoxetine by irradiation disrupted its behavioral consequences, thus suggesting that neurogenesis is required for the behavioral effects of antidepressants (Santarelli et al., 2003).

Following this seminal paper, studies from the same group and others brought controversies in the conclusion. Thus the same authors administered fluoxetine to Balb/cJ mice, a strain prone to anxiety that is used to develop behavioral tests of antidepressants sensitive to chronic but not acute treatment, as observed in humans. They reported that irradiation did not prevent the behavioral effects of fluoxetine while it dramatically reduced adult neurogenesis by ablating progenitor cells. As this result was in opposition to their previous observations, they checked many alternative explanation to conclude that the differences in response to chronic fluoxetine were linked to the strain of mice used and not the tests and paradigms used (Holick et al., 2008).

Although this seems conceivable, a thorough reading of their first study indicated that in order to reach this conclusion, they had also used mice from the Balb/cJ strain and reported the same global observations: irradiation, which blocks cell proliferation, prevented the action of fluoxetine on behavioral responses to chronic unpredictable stress, leaving the question of Balb/cJ mice neurogenic sensitivity to fluoxetine unsolved. However, contemporaneous papers confirmed that although Balb/cJ mice respond behaviorally to chronic fluoxetine treatment, they do not display any increase in neurogenesis (Huang et al., 2008), confirming the assumption of strain differences in sensitivity to fluoxetine, and indicating that neurogenesis may not always be required for the behavioral effects of fluoxetine, which appear to be strain dependent.

Confirming these indirect conclusions, a study by Navailles and collaborators directly compared neurogenic responses of Balb/cJ and C57Bl/6J mice to fluoxetine treatment during adolescence and found that although both strains reacted with an initial increase in cell proliferation, the expected increase in 2 weeks old cells observed in C57Bl/6J was totally absent in Balb/cJ mice, suggesting that the newborn cells did not survive in this strain (Navailles et al., 2008).

Altogether, although they have not been exploited in this direction so far, these strain differences are extremely interesting

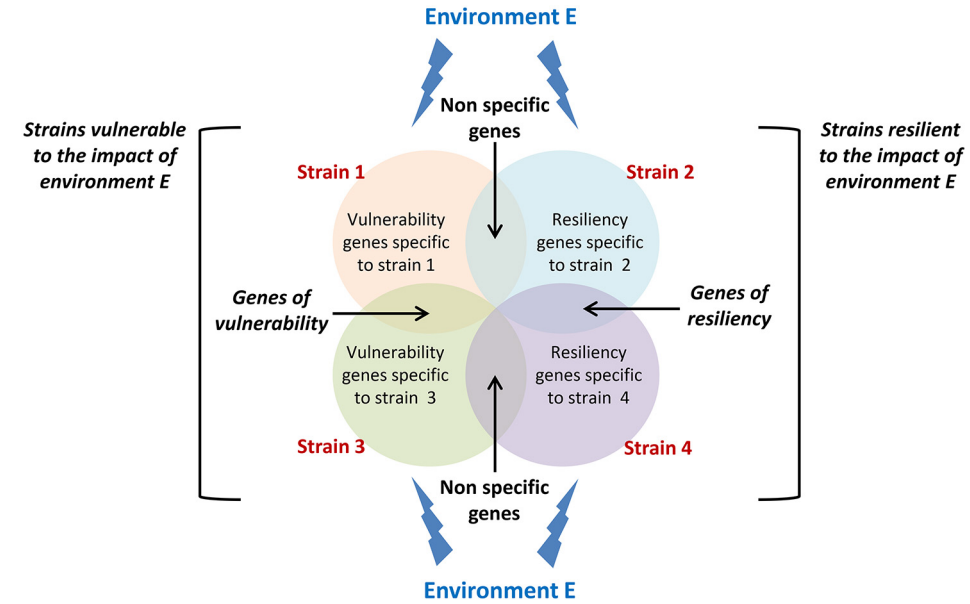


**FIGURE 2 | Conceptual framework for isolating vulnerability and resiliency genes.** Circles represent genes which expression changes in response to a specific environment E in different strains displaying either vulnerability (strain 1 and strain 3) or resiliency (strain 2 and strain 4) to this environment. Genes that expression varies in both vulnerable and resilient strains cannot sustain vulnerability or resiliency and should be disregarded. Genes that expression varies in both vulnerable strains and not in resilient strains are potential genes sustaining vulnerability to environment E. The same applies to resiliency genes.

if one considers that responsiveness to antidepressants is highly variable among patients and that this strain difference certainly has clinical relevance. We thus propose to complete Holick and colleagues statement that "***it is imperative that future studies utilize rodent behavioral models sensitive to chronic antidepressant treatment to dissect which of these neural changes play a causal role in their behavioral effects***" (Holick et al., 2008) by adding that it is also imperative to thoroughly compare sensitive and insensitive strains to dissect the neural changes induced by treatment that are specifically relevant for their behavioral consequences.

## Conclusion

The latent idea behind gene-environment interaction studies is to identify molecular or neurobiological factors common to high responding strains and uncommon to non-responding strains that are capable of governing the genesis of new neurons in the adult hippocampus. As this field of research is still at its premises, studies so far have mainly demonstrated the existence of a complex interaction between genetic constraints and life events but none of them has yet identified the mechanisms involved. A proposed setup for future studies would be to analyze changes in gene expression in strains showing sensitivity and resistance to a specific environmental stimulation (**Figure 2**) in order to isolate those changes that specifically support vulnerability and resiliency of adult neurogenesis to environmental challenges.

Overall, data presented in this review thus indicate that in addition to genes that have been isolated for their control of the different phases of neurogenesis, the regulation of adult neurogenesis is also governed by a set of genes that confer to an individual an increased sensitivity or a certain resistance to life events. Isolating these genes (**Figure 2**), the mechanisms by which environmental factors can affect their expression, and the mechanisms by which they regulate adult neurogenesis will constitute major steps in our understanding of individual differences in adult neuroplasticity.

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# The interplay of early-life stress, nutrition, and immune activation programs adult hippocampal structure and function

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Early-life adversity increases the vulnerability to develop psychopathologies and cognitive decline later in life. This association is supported by clinical and preclinical studies. Remarkably, experiences of stress during this sensitive period, in the form of abuse or neglect but also early malnutrition or an early immune challenge elicit very similar long-term effects on brain structure and function. During early-life, both exogenous factors like nutrition and maternal care, as well as endogenous modulators, including stress hormones and mediator of immunological activity affect brain development. The interplay of these key elements and their underlying molecular mechanisms are not fully understood. We discuss here the hypothesis that exposure to early-life adversity (specifically stress, under/malnutrition and infection) leads to life-long alterations in hippocampal-related cognitive functions, at least partly via changes in hippocampal neurogenesis. We further discuss how these different key elements of the early-life environment interact and affect one another and suggest that it is a synergistic action of these elements that shapes cognition throughout life. Finally, we consider different intervention studies aiming to prevent these early-life adversity induced consequences. The emerging evidence for the intriguing interplay of stress, nutrition, and immune activity in the early-life programming calls for a more in depth understanding of the interaction of these elements and the underlying mechanisms. This knowledge will help to develop intervention strategies that will converge on a more complete set of changes induced by early-life adversity.

**Keywords: hippocampus, neurogenesis, cognition, early-life stress, early-life nutrition, early-life neuroimmune activation, early-life infection**

## INTRODUCTION

Clinical studies have provided evidence that cognition in later life is strongly influenced by experiences occurring during the sensitive period of early development and adolescence. Indeed, adverse early-life events, e.g., social deprivation or abuse, are associated with an increased vulnerability to develop psychiatric disorders (Stevens et al., 2008; Maselko et al., 2011) and impaired cognitive functioning in adulthood (Chugani et al., 2001; Kaplan et al., 2001; Nelson et al., 2007; Mueller et al., 2010). Interestingly, exposure to prenatal or postnatal malnutrition, e.g., the lack of one or multiple essential nutrients, can lead to a similarly increased incidence of psychopathologies (Brown et al., 2000; Costello et al., 2007; Mueller et al., 2010) and cognitive deficits in adolescence and adulthood (Walker et al., 2000; Benton, 2010; de Rooij et al., 2010; de Groot et al., 2011; de Souza et al., 2011; Laus et al., 2011).

Different lines of work further illustrate the relation between cognitive functions and postnatal immune system activity. For example, maternal inflammatory responses during pregnancy (Eriksen et al., 2009), antenatal infection in preterm babies (Dammann et al., 2002; Eriksen et al., 2009; van der Ree et al., 2011) and neonatal infection (Rantakallio et al., 1997; Libbey et al., 2005) are suggested as risk factors for a lower IQ later in life and for an increased vulnerability to develop later neuropsychiatric disorders like schizophrenia and depression (Brown, 2006; Cope and Gould, 2013; Miller et al., 2013; O'Connor et al., 2014). These lines of research support the hypothesis postulated in the developmental origins theory of Barker that the early-life environment determines the framework for later adult functioning and the development of pathologies through an early programming of adult brain structure and function (Barker, 2004; Barker et al., 2013). Although the consequences of an adverse early-life history for adult cognitive function are confirmed by evidence from preclinical studies in rodents (Lucassen et al., 2013; Naninck et al., 2013), the exact mechanisms underlying these programming effects and the determining elements in the environment that modulate these processes remain obscure. As is clear from the above examples, early-life environmental experiences cannot be avoided (Danese et al., 2009; Bale et al., 2010) and it is thus crucial to understand which elements play a role, and how they interact in order to develop future interventions.

The early postnatal environment encompasses many essential elements, which are key and determinant for proper brain development, many of which are largely transmitted via the mother–child interaction. A child is generally dependent on maternal care during the first weeks of life, encompassing tactile stimulation,

nutritional provision, as well as transfer of antibodies and maternal warmth. As is evident from the above examples, an adverse early-life environment may affect the stress hormones, nutrition, or inflammatory modulators, all elements that can strongly interact and affect one another (Kelly and Coutts, 2000; Shanks and Lightman, 2001; Miller et al., 2009; Palmer, 2011). Hence, an individual is actually exposed to a combination of these factors rather than to one of these elements independently.

There is, e.g., increasing evidence that early-life maltreatment is associated with an increase in the pro-inflammatory markers of the immune system in adulthood (Coelho et al., 2014). Also, associations have been found between maternal supplementation of specific nutrients (e.g., folate, iodine, and vitamin D) and an enhanced fetal immune system development that was paralleled by a reduced incidence of psychopathologies in adulthood (Marques et al., 2013). Also, Monk et al. (2013) reviewed the interrelation of nutrition and prenatal stress in stress-induced maternal malnutrition. An interrelated profile of different early-life elements brings forward the possibility of confounding factors in the currently reported findings on the possible mechanisms that underlie such programming. Interestingly, most studies addressing the mechanisms underlying early-life programming consider these elements individually, but, considering that each one of these elements has a very strong interaction with one another, and influence each other greatly, it is likely that the final effect will be determined by the synergistic action of the different early-life elements at play. Hence, it will be necessary to re-discuss and re-analyze the so far obtained results in light of such interactions.

The focus of this review will be on the essential elements present in the early postnatal environment and their involvement in the lasting effects on cognitive functioning. In particular we will discuss how the various elements during the early postnatal period (i.e., sensory stimuli, nutrition, stress hormones, and inflammatory molecules) interact, affect each other and ultimately how they may synergistically affect brain structure, and function on the long term. We focus on the consequences for hippocampal structure and related cognitive functioning and on a unique form of hippocampal plasticity, adult neurogenesis. The hippocampus is one of the key brain regions important for cognitive functions and this form of plasticity is very important for learning and memory processes and highly modulated by (early) environmental factors, i.e., stress (Gould et al., 2000; Mirescu et al., 2004; Dranovsky and Hen, 2006; Lucassen et al., 2013), nutrition (Lindqvist et al., 2006; Beltz et al., 2007; Coupé et al., 2009), and immune activation (Das and Basu, 2008; Green and Nolan, 2014; Musaelyan et al., 2014).

The fact that the hippocampus is particularly susceptible to influences of the early-life environment and in particular stressful stimuli is easily understood when considering the important developmental processes that take place in this brain region during this sensitive developmental period. Indeed, hippocampal and dentate gyrus (DG) development in particular starts during late gestation and continues during the first 2 weeks after birth (Altman and Bayer, 1990), while in human the development of the DG starts during the last trimester of pregnancy and continues to about 16 years of age (Arnold and Trojanowski, 1996). During this time, granular cells are generated in the subventricular zone or in the hippocampus itself, that migrate to the different layers of the DG (Pleasure et al., 2000; Fukuda et al., 2005; Navarro-Quiroga et al., 2006), while in adulthood quiescent neuronal progenitors cells develop to become functional granular cells (Kempermann et al., 2004). Interestingly, adult neurogenesis is lastingly affected by perturbation of the early-life environment as well (Coupé et al., 2009; Oomen et al., 2011; Korosi et al., 2012; Lucassen et al., 2013; Loi et al., 2014; Musaelyan et al., 2014). Next to the generation and migration of granular cells, the migration and colonization by microglia takes place as well during this sensitive period and is also peeking in the first few postnatal days (Schwarz and Bilbo, 2012; Schwarz et al., 2012; Cope and Gould, 2013). These migratory processes are supported by a scaffold formed by immature astrocytes. In addition, glia cells are increasingly acknowledged for there role in the plasticity and circuit functioning of the adult hippocampus (Allen and Barres, 2009; Ekdahl, 2012). Finally, the hippocampus is highly sensitive to stress both early as well as during adult life due to its remarkably high expression levels of the glucocorticoid receptor (GR). Interestingly, expression levels of these receptors have been shown to be affected by early-life stress as well (Liu et al., 1997; Lucassen et al., 2013; de Kloet et al., 2014).

In the following sections we discuss the effects of early-life stress, nutrition, and central immune activity on hippocampal function and adult neurogenesis and thereafter discuss how to implement in these findings that (1) these elements affect one another and (2) they act synergistically to exert their function.

## MODULATION OF HIPPOCAMPAL FUNCTION AND NEUROGENESIS BY EARLY-LIFE STRESS

Early-life stress exposure is strongly associated with cognitive impairments later in life (Kaplan et al., 2001; Stevens et al., 2008; Mueller et al., 2010; Maselko et al., 2011), but what is the preclinical evidence for this association? Which are the most common animal models to study this question?

Stress in the postnatal period can be induced using several rodent paradigms. The most widely studied models to induce early-life stress involve either naturally occurring variation or artificial modulation of maternal care (Francis and Meaney, 1999; Francis et al., 2000), repeated dam-litter separation or a single prolonged deprivation of the dam and her pups (Schmidt et al., 2010; Hedges and Woon, 2011) and chronic early-life stress (Ivy et al., 2008; Rice et al., 2008; Wang et al., 2011) during the first few postnatal weeks. Adult offspring from all of these early-life stress models exhibit cognitive impairments, indicating a strong translational value of these models in addressing the underlying mechanism of such programming. For example, adult rats that had been either maternally separated during the first 2 weeks of life or deprived on postnatal day (P)3 for 24 h, exhibited impairments in their acquisition of spatial information in the Morris water maze (MWM; Oitzl et al., 2000; Huot et al., 2002; Aisa et al., 2007; Fabricius et al., 2008; Oomen et al., 2010) and mice exposed to chronic early-life stress show impairment of spatial memory (tested by MWM and object location) and declarative memory tested by novel object recognition task and Y-maze (Rice et al., 2008; Wang et al., 2011). These early-life stress induced cognitive impairments are associated with a number of alterations in

hippocampal structure and neuronal plasticity, including decrease in dendritic complexity and spine density in the cornu ammonis (CA)1 and CA3 (Huot et al., 2002; Ivy et al., 2008; Wang et al., 2011), reduced DG dendritic complexity, granular cell number and granular cell density (Oomen et al., 2010, 2011), reduced astrocyte density in the DG and CA regions (Leventopoulos et al., 2007), and age-dependent alterations in adult hippocampal neurogenesis levels (Korosi et al., 2012). Offspring in both rats and mice exposed to a form of early-life stress exhibit a short-term increase followed by a permanent reduction of proliferating and immature cells (Mirescu et al., 2004; Nair et al., 2007; Oomen et al., 2009, 2010; Hulshof et al., 2011; Suri et al., 2013; Naninck et al., 2014). Levels of cell survival and astrogenesis in young adults are generally not affected after maternal separation (Oomen et al., 2010; Hulshof et al., 2011; Kumar et al., 2011; Suri et al., 2013), but this is strongly reduced at a more advanced age both in rats exposed to low levels of maternal care (Bredy et al., 2003), as well as in mice exposed to chronic early-life stress (Naninck et al., 2014).

How exactly does early-life stress exposure lead to the above described lasting alterations in cognitive functions as well as hippocampal structure and function remains relatively uncertain, but several possible mechanisms of action have been identified over the last years. First, available evidence that early-life stress manipulations, including maternal separation, maternal deprivation or chronic stress via environmental manipulation, alter the quality and/or quantity of maternal care (Brown et al., 1977; Pryce et al., 2001; Macrì et al., 2004; Brunson, 2005; Fenoglio et al., 2006; Korosi and Baram, 2010) strongly suggests that mother–infant interaction is crucial in programming brain and behavior. However, these studies do not directly address whether maternal care, under normal conditions, is actively involved in these regulations.

In support of this notion, it has been demonstrated that a natural variation in maternal care (Liu et al., 1997; Champagne et al., 2008; Bagot et al., 2009) and individual within-litter variation in the amount of active care received (van Hasselt et al., 2012a,b) leads to differences in stress response and cognitive functions associated with altered hippocampal structure, plasticity and changes in the neuroendocrine system in later life. In line with this evidence from animal studies, in pre-term and term neonates, different forms of sensory stimulation, such as moderate touch or skin-to-skin care, have been shown to have beneficial consequences, including reduction of pain responsiveness in neonates (Cignacco et al., 2007) and a reduced reactivity to stress (Feldman et al., 2010). While it is clear that sensory stimuli that the mother gives to her offspring is highly dependent on the well-being of the mother and offspring and thus strongly affected by stressful environment, how exactly this element interacts with the other key elements in the early environment to lead to the programming of the brain structure and function is yet unclear. The few studies tackling the interaction of sensory stimuli with either nutritional or immune challenges are discussed in Sections "The Interplay of the Different Elements in the Early-Life Environment" and "Early-Life Adversity; Opportunities for Intervention Later in Life."

The role of stress-related hormones (corticosterone; CORT) and neuropeptides (e.g., corticotropin releasing hormone, CRH) in the modulation of early-life stress effects on the hippocampus has been studied extensively. When the HPA axis is activated by a stressor this leads to HPA axis activation, which in turn leads to the initial release of CRH from the hypothalamic paraventricular nucleus (PVN), stimulation of pituitary adrenocorticotropic hormone (ACTH) secretion into the blood, and the subsequent release of glucocorticoids from the adrenal glands: CORT in rodents and cortisol in humans. Negative feedback takes place when glucocorticoids bind to GRs in the hippocampus, PVN, prefrontal cortex and pituitary and thereby inhibit release of CRH and ACTH (Tsigos and Chrousos, 2002).

In fact, exposure to stress during the postnatal early-life period programs the basal and stress-induced activation of the HPA axis and the behavioral responses to stress throughout life (Seckl and Meaney, 2006; Heim and Binder, 2012). Importantly during the first 2 weeks of life, the stress response is believed to be hyporesponsive to some, but not all stressors. This consists of a smaller, or absence of HPA axis responsiveness in pups when compared to the adult organism (Stanton et al., 1988; Levine et al., 1991). Age appropriate stressors, like maternal deprivation or fragmented maternal care elicit secretion of CORT (Yi and Baram, 1994; Schmidt et al., 2004; Rice et al., 2008). There is increasing evidence that next to an increased release of CORT (basally and upon stress exposure), the expression levels of several of the genes involved in the modulation of the stress response (e.g., CRH and GR; Rice et al., 2008; Ivy et al., 2010; Wang et al., 2011; Chen et al., 2012) are lastingly altered in offspring experiencing early-life stress. Thus it is reasonable to assume that these changes might be (at least partly) mediating the altered hippocampal plasticity and thereby the associated cognitive impairments. However, the lasting alterations in the levels of circulating CORT are not consistently permanent in different models of early-life stress (Mirescu et al., 2004; Brunson, 2005; Rice et al., 2008).

While glucocorticoid exposure during early-life evoked cognitive impairments in adulthood (Kamphuis et al., 2003) and there is abundant literature about the regulating (mostly inhibiting) role of CORT (during adulthood) on neurogenesis (Cameron and Gould, 1994; Lucassen et al., 2010), chronic depletion of CORT through adrenalectomy of rats at 10 days of age did, however, not induce alterations in neurogenesis in adulthood (Brunson et al., 2005). Whether the raise in CORT early in life (and no longer in adulthood) modulates the process of neurogenesis and cognitive impairments on the long-term thus remains to be determined. The paucity of data points to the need for further research in this area; however, the contradictory data from the existing studies suggest that other factors may also contribute to the mechanisms by which early-life experience programs brain structure and function.

Because CRH expression is permanently altered after early stress in various models in the hypothalamus (Plotsky and Meaney, 1993; Liu et al., 1997) and hippocampus (Ivy et al., 2010), CRH has been explored as a modulator for the consequence of early-life stress in the hippocampus (Brunson, 2005; Ivy et al., 2010; Korosi et al., 2010; Wang et al., 2011; Loi et al., 2014). Indeed, exposure to CRH can mimic the changes in hippocampal structure induced after chronic early-life stress (Brunson, 2005) and a selective blockage of the CRF receptor type 1 during the first

week after chronic early-life stress indeed prevented the apparent cognitive impairments in the early-life stressed animals (Ivy et al., 2010). Intriguingly, conditional CRF1 knock-out mice were 'protected' against the hippocampus dependent cognitive impairments induced by chronic early-life stress (Wang et al., 2011). Finally early-life stress has been shown to age-dependently affect the expression of the gene and protein of the neurogenic factor brain derived neurotrophic factor (BDNF; Nair et al., 2007; Zimmerberg et al., 2009; Suri et al., 2013). Indeed BDNF expression and concomitant levels of hippocampal neurogenesis are upregulated by early-life stress during development and young adulthood, but reduced to decreased levels with aging (Nair et al., 2007; Suri et al., 2013; Naninck et al., 2014). These data suggests a possible role of BDNF in the modulation of hippocampal plasticity after early-life adversities.

Summarizing, stress related hormones and neuropeptides are involved in mediating lasting effects of early-life stress, however they are not sufficient to explain all the observed effects, indicating that other factors, possibly acting synergistically with these stress molecules, are also involved in the programming. We will next explore the role of nutrition early in life in these processes.

## EARLY NUTRITIONAL FACTORS DETERMINE ADULT HIPPOCAMPAL STRUCTURE AND FUNCTION

As mentioned in the introduction, early nutritional insults have lasting consequences for brain development and function later in life (Lucas, 1998; Brown et al., 2000; McMillen et al., 2008; Prado and Dewey, 2014) with later cognitive functions being particularly affected (de Groot et al., 2011). This is not surprising when considering that during the first postnatal period, the brain is under heavy development and an incredible nutritional demand. In fact, for proper brain development to occur, specific dietary macro-, and micronutrients are essential during gestation and lactation (Scholl et al., 1996; Benton, 2010; Dangat et al., 2010; Veena et al., 2010). Thus, disruption of the nutritional supply (quality and quantity) to the offspring will have major effects on the development of the brain, and more specifically on the hippocampus. An inadequate supply of them during critical developmental periods leads to brain dysfunction and cognitive impairments later in life (McNamara and Carlson, 2006; Innis, 2008; de Groot et al., 2011; Laus et al., 2011).

The offspring is during this critical developmental period fully dependent on the nutrition provided by the mother. Most preclinical models are based upon altering maternal nutrition during gestation and/or lactation. Indeed, micronutrient composition of the maternal diet during gestation and lactation determine the balancing of fatty acid (FA) levels in the brain of the offspring, as maternal micronutrients (Roy et al., 2012) affect the breast milk composition (Innis, 2008; Allen, 2012). The association of early-life nutrition and cognitive functions is further supported by preclinical evidence (Campbell and Bedi, 1989; Castro et al., 1989; de Souza et al., 2011; Valladolid-Acebes et al., 2011; André et al., 2014). Here, we will discuss how early postnatal nutritional stress and specific nutritional components present in the postnatal period modulate cognition and neurogenesis in adulthood.

Various models are used to study how early malnourishment affects brain development and cognitive functions, e.g., through dietary restriction, overnutrition, or malnutrition by limitation of different key elements during gestation and/or lactation. For instance, protein restriction, global dietary restriction to 50% or high-fat, and modulation of essential macro- and micronutrients that need to be obtained from the diet are commonly used approaches (Campbell and Bedi, 1989; Bedi, 1992; Martinez et al., 2009; Valadares et al., 2010; de Souza et al., 2011; Roy et al., 2012). For example, protein restriction during lactation in rats (Valadares et al., 2010) and 12 h restriction of maternal milk (Castro et al., 1989) evokes deficits in hippocampus dependent spatial memory tested by MWM and object recognition in adult offspring, but see (Wolf et al., 1986; Campbell and Bedi, 1989). These deficits are accompanied by alterations in hippocampal structure and plasticity as well. Food restriction to 50% of the normal intake during lactation changed the time course of BDNF production and proliferation in the hippocampus (Coupé et al., 2009) and reduced the number of proliferating cells in the adult offspring, without affecting cell survival or cell fate (Matos et al., 2011). Furthermore, protein restriction during the same period leads to reduced total granular cell numbers (Bedi, 1991) and food restriction to 50% during gestation and lactation reduced hippocampal volume (Katz et al., 1982). In addition to these nutritional restriction studies, the offspring of high-fat diet exposed dams exhibit a reduction in postnatal neurogenesis during development (Tozuka et al., 2009) and impaired dendritic differentiation of newborn neurons in the adult hippocampus (Tozuka et al., 2010). However, to date no further studies of adult hippocampal neurogenesis in early-life food-restricted or high-fat diet exposed animals have been performed.

The lipid content during early-life is essential for the composition of maternal milk during lactation and development of the pup brain. For instance, polyunsaturated fatty acids (PUFAs), including the omega-3 FA docosahexaenoic acid (DHA) and omega-6 FA arachidonic acid (AA), are structural components of the brain that promote healthy neuronal growth, repair, and myelination (McNamara and Carlson, 2006). Deficiency of these FAs in the maternal diet first revealed the association of low FA composition and impaired learning and memory functions (Lamptey and Walker, 1978). Moreover, deficiency of omega-3 FA during gestation and lactation impairs the spatial memory (tested by Barnes maze; Fedorova et al., 2009), whereas artificial feeding of rats with omega-3 FA deficient milk during lactation prolonged escape latency in the MWM (Lim et al., 2005) and omega-3 FA enrichment improved performance of the animals (Carrié et al., 2000).

These functional changes following FA deficiency are furthermore associated with structural changes in the brain. Maternal omega-3 FA deficiency during gestation leads to underdevelopment of the primordial hippocampus in fetal rats at the last days of gestation (Bertrand et al., 2006). Nutritional omega-3 FA deficiency during gestation and lactation reduces pyramidal cell size in the hippocampus (Ahmad et al., 2002) and the levels of markers for neuronal plasticity such as BDNF (Madore et al., 2014) at weaning. In addition, dietary enrichment with omega-3 prevents the adverse consequences of early-life sevoflurane (anesthesia) exposure on cell proliferation in the hippocampus and the induced memory impairments (Lei et al., 2013). Maternal supplementation

of α-linolenic acid (ALA), a precursor of omega-3 FA, during gestation and lactation enhanced hippocampal neurogenesis at P19 (Niculescu et al., 2011). However, to date it has not been studied whether an imbalance of FAs in early-life lastingly affects adult hippocampal neurogenesis.

Next to essential FAs, essential amino acids, choline, and methionine, and micronutrients such as folic acid (B9), vitamin B6 and B12, are also essential for brain development (Roy et al., 2012). Deficiency of choline during gestation and lactation impairs working memory in the 12-arm spatial memory maze, while supplementation enhances performance (Meck and Williams, 1999). In addition, a deficiency of nutritional folate, choline, B6, and B12 during gestation and lactation leads to learning and memory impairments in the radial arm mazes and enhanced the number of apoptotic cells in the hippocampus (Blaise et al., 2007). Deficiency of these methyl donors furthermore affects hippocampal neurogenesis by altering the apoptotic rate (Craciunescu et al., 2010).

Summarizing, evidently the nutritional composition during critical developmental periods (pre and postnatal) of life is essential for the proper development, structure and function of the hippocampus. Similar to the cognitive impairments induced by early-life stress, early-life malnutrition evokes such deficits as well. Another important element known to play a key role in modulating brain development and function is the neuroimmune system, which will be discussed in the next section.

## EARLY-IMMUNE RESPONSE ACTIVATION PROGRAMMING THE LATER-LIFE HIPPOCAMPUS

Activation of the peripheral and/or central immune system in early-life is associated with psychopathologies in adulthood, including cognitive dysfunction (Rantakallio et al., 1997; O'Connor et al., 2014). For instance, maternal infection during pregnancy is associated with lower IQ in adult men (Eriksen et al., 2009). In addition, pre- and postnatal infection have been associated with anxiety-like and depressive-like behavioral responses and cognitive impairments in adolescence and adulthood (Das and Basu, 2011; Williamson et al., 2011; Doosti et al., 2013; Dinel et al., 2014). The modulating effects of early-life immune challenges on brain function are not unexpected considering the essential role of neuroimmune cells in (early-)life. Microglia and astrocytes mediate many processes in the brain, including neuroinflammatory responses (Capuron and Miller, 2011; Green and Nolan, 2014; O'Connor et al., 2014), neuronal activation and plasticity (Slezak et al., 2006; Halassa et al., 2009; Parpura et al., 2012; Greter and Merad, 2013), maintenance and development of the blood–brain barrier (BBB; Banks et al., 1995; Chaboub and Deneen, 2013) and importantly, neurogenic processes during development (Das and Basu, 2011; Schwarz and Bilbo, 2012; Cope and Gould, 2013) and adulthood (Das and Basu, 2008; Ekdahl, 2012; Cope and Gould, 2013; Cunningham et al., 2013; Kohman and Rhodes, 2013; Sierra et al., 2013, 2014). Thus, imbalanced activation of the microglia, in particular during early-life, has the potential to lastingly disturb internal homeostasis and proper brain development (Allen and Barres, 2009).

Activity of the microglia is controlled by immune response regulating effector molecules, like pro-inflammatory (e.g., IL-1β, IL-6, and TNFα) and anti-inflammatory (e.g., IL-4 and IL-10) cytokines or chemokines (Chaplin, 2003; Cartier et al., 2005; Ekdahl et al., 2009) that regulate the communication between immune cells in the peripheral and central immune system. Although the brain is a relatively concealed and immunosuppressed environment in adulthood, that is separated from the periphery by the BBB, cytokines, and chemokines in the periphery have the potential to cross the BBB and can affect the innate cells of the brain (Banks et al., 1995; Pan and Kastin, 1999). Interestingly, during early-life, peripheral immune challenges might have a greater potential to adversely affect the brain (Schoderboeck et al., 2009). During this time, the BBB still pre-exists in a leaky stage till a few days after birth (Engelhardt, 2003), providing the possibility of a greater immunoreactive responses in the brain when a peripheral infection occurs. In addition, microglia develop in close parallel to developmental neurogenesis and appear in an activate and amoeboid state during development, whereas they are present as resting, ramified cells in adulthood (Bilbo and Schwarz, 2009).

In the following part, we will discuss the pre-clinical evidence in support of a direct role of postnatal immune challenges in the persistent modulation of hippocampal structure and function. Most studies of postnatal infection have focused on stimulation by bacteria like *Escherichia coli* or the Gram-negative bacteria component lipopolysaccharide (LPS). We will here address the hippocampus dependent cognitive functions following early-life neuroimmune stress from two different angles. Firstly, activation of the peripheral neuroimmune system and its immediate and lasting effects on central neuroimmune system function and brain function. Secondly, the consequences of central neuroimmune system activity without a prior peripheral immune challenge, for instance via activation of central viral infection or pro-inflammatory factors, on hippocampal function and neurogenesis in adulthood.

A peripheral immune challenge with LPS (P1) or *E. coli* (P4) in the rat pup elicits an elevation of pro-inflammatory cytokines and CORT in the first few hours after the challenge in blood serum (Bilbo et al., 2005b), whole brain (Ortega et al., 2011) or hippocampus (Bilbo et al., 2005b; Dinel et al., 2014). In adulthood, cytokine mRNA expression levels in the brain of the early-infected animals remain normal under basal conditions, with the exception of elevated hippocampal levels of TNFα (P4 infected; Bland et al., 2010a) and IL-1β (P5 LPS infected; Wang et al., 2013). Although overall no strong changes in the cytokine expression profiles are present in the adult, in the early-life infected animals, microglial activation markers indicate enhanced reactive microglia (CD11b+) in the hippocampus (Bilbo et al., 2005a) and CA1 region (Iba1+; Bland et al., 2010b). In addition, adult rats exposed to LPS at P3 and P5 exhibited a hippocampus-specific increase in Iba1+ immunoreactive microglia in the CA1 and DG (Sominsky et al., 2012), indicative of a priming effect on hippocampal microglia. Indeed, a peripheral LPS injection in adulthood exerts an exaggerated pro-inflammatory cytokine response of mainly IL-1β in the hippocampal CA1 of rats with a history of early-life infection (Bilbo et al., 2005a, 2008), probably evoked by a programmed pro-inflammatory response of hippocampal microglia.

Interestingly, the pro-inflammatory response in the hippocampus following peripheral infection is accompanied by a direct effect on developmental hippocampal neurogenesis and structural changes in adulthood. *E. coli* infection at P4 immediately suppresses gene expression of neurotropic factor BDNF in the CA1 and CA3 (Bilbo et al., 2008) and neuronal and astrocytic cell proliferation is reduced in the hippocampus following LPS exposure at P9 (Järlestedt et al., 2013). Hippocampal cell proliferation then restores to normal conditions in the 48 h after the challenge and does not affect the survival of immature neurons during the time of infection (Bland et al., 2010a; Järlestedt et al., 2013). This developmental change probably underlies the reduction in later hippocampal volume observed in early-life infected adult rodents (Wang et al., 2013). Moreover, in adulthood, early-life *E. coli* infected rats and LPS infected mice have comparable numbers of dividing, differentiating, and surviving neurogenic cells in the subgranular zone as control animals (Bland et al., 2010b; Järlestedt et al., 2013; Dinel et al., 2014). In conclusion, neurogenesis does not seem to be heavily affected by peripheral immune challenges. These subtle changes in neurogenesis are in line with the findings of limited effects on hippocampus-related cognitive functioning as well. Most studies of early-life infection do not find changes in different learning and memory paradigms, such as fear conditioning, MWM or Y-maze (Bilbo et al., 2005a, 2007, 2008; Dinel et al., 2014), but see (Harré et al., 2008; Wang et al., 2013). Interestingly, however, a stronger modulation of hippocampus-related cognitive functions is manifested after a second immunological challenge in early-life infected rodents. Thus, a combination of early-life infection history and adolescent or adult LPS (re-)exposure evokes impairments in a contextual fear conditioning paradigm (Bilbo et al., 2005a, 2006) and spatial memory performance in the Y-maze (Dinel et al., 2014), but not in the MWM (Bilbo et al., 2007).

Interestingly, these cognitive impairments in response to a second immune challenge appear in accordance with reduced newborn cell survival in the early-infected rats (Bland et al., 2010a), but not with BDNF gene expression levels (Bilbo et al., 2008). In contrast, neurogenesis is upregulated after LPS during adulthood in animals that never had an immune challenge before (Bland et al., 2010a). The consequences of a second immunological challenge in adulthood might thus be resulting from the priming effects of an early-life infection on the population of adult hippocampal microglia. This may lead to an exaggerated pro-inflammatory response with detrimental effects on adult hippocampal neurogenesis and cognition.

Neuroimmune system activation is not solely induced by peripheral bacterial components. The consequences of direct modulation of central cytokines and/or central induction of innate immune cells on the hippocampus are moderately studied. An example is TNFα injection at P3 and P5, increasing anxiety-like behaviors in male mice (Babri et al., 2014b). However, other cytokine overexpression levels have not been elegantly studied. For some years now, viral infections have been considered a contributing factor to the development of neuropsychological disorders, including hippocampal related dysfunction (Das and Basu, 2011). Various viruses are used to investigate disruption in hippocampal development and adult neurogenesis, e.g., the lymphocytic choriomengitis virus (LCMV) (Pearce et al., 1996; Sharma et al., 2002; Orr et al., 2010), Borna disease virus (BDV; Zocher et al., 2000; Sauder et al., 2001) or polyinosinic:polycytidylic acid (Poly I:C; Galic et al., 2009). Each of these viruses induces different phenotypical changes. Poly I:C intracerebroventricular injection at P14 induces short time elevations of pro-inflammatory cytokine IL-1β in the hippocampus and adult-onset deficits in contextual fear conditioning (Galic et al., 2009). But earlier central administration of LCMV at P4 produces lasting IL-1β induction with subsequent loss of cells in the granular cell layer (Sharma et al., 2002; Orr et al., 2010) and reduced levels of progenitor cells in the DG (Sharma et al., 2002), without affecting other hippocampal regions (Pearce et al., 1996). This virus induced phenotype could be reduced by the use of an anti-inflammatory agent to block IL-1β, which restored the granular cell numbers in adulthood (Orr et al., 2010). The BDV virus typically induces apoptosis of DG cells at 27 and 33 days post infection, possibly mediated by a reduction in neurotrophins in this brain region (Zocher et al., 2000) and further impairs MWM performance in adulthood, correlating to chemokine expression levels (Sauder et al., 2001). How these virus infections early in life mechanistically affect the hippocampus is unfortunately poorly understood to date (Das and Basu, 2011).

Altogether, early-life peripheral infection immediately increases pro-inflammatory cytokines in the hippocampus and exerts lasting effects on hippocampal structure, but evokes only subtle alterations in hippocampal neurogenesis and functionality under basal condition. After exposure to a second immunological challenge in adulthood, however, a history of early-life infection has aversive effects on cognitive function related to an exaggerated pro-inflammatory response in the hippocampus. On the other hand, viral infection that induces a central stimulation of the immune system leaves detrimental effects on the DG, affecting adult hippocampal neurogenesis and cognitive functions. The lasting effect of early-life infection on hippocampal microglia suggests that a programming effect of peripheral and ultimately central immune system activity plays an important role in the lasting effects of hippocampal structure and cognitive functions.

## THE INTERPLAY OF THE DIFFERENT ELEMENTS IN THE EARLY-LIFE ENVIRONMENT

The discussed consequences of early-life stress, nutrition, and immune activation can all be considered forms of early-life adversity. Although limited studies have examined the integrated role of these elements, the presented evidence in the above sections clearly points to the fact that challenges, even when very different in nature (disruption of maternal care, malnutrition, or immune), lead to strikingly similar outcomes of disrupted hippocampal structure and plasticity later in life as well as cognitive impairments. Knowing that these systems are tightly related and that they affect each other, it is reasonable to assume that the current models of early-life stress, malnutrition and infections discussed up to now elicit effects on all these different levels (**Figure 1**) and that it is the synergistic effects of all of these components that lead to the observed outcome rather than only the experimentally modulated one. In the upcoming section, we will discuss the current evidence and missing links for this hypothesis. Because the

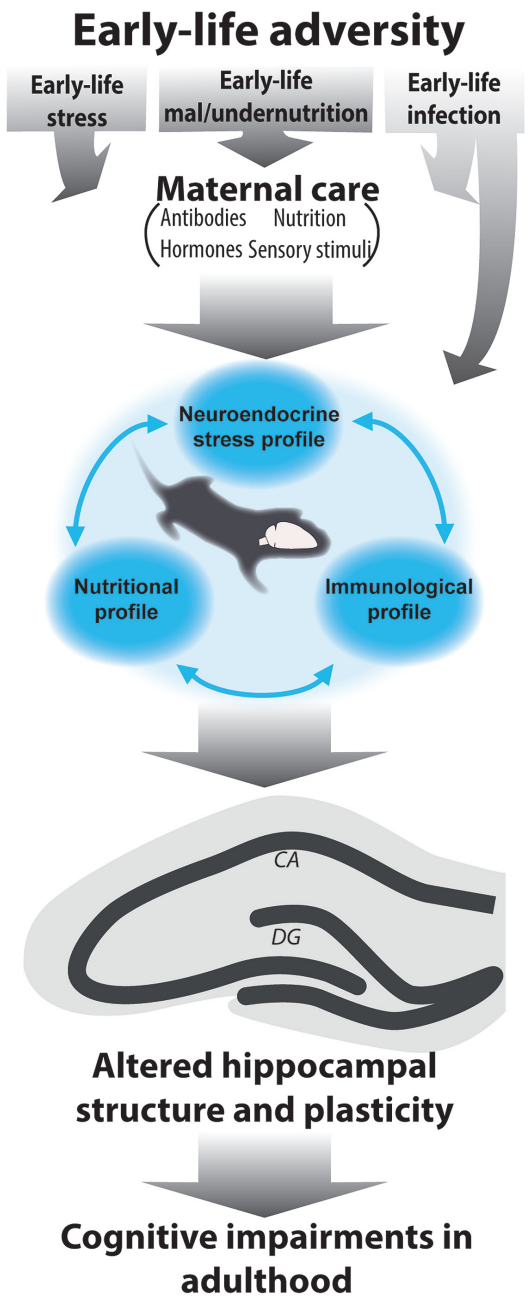


**FIGURE 1 | Schematic representation of the interrelated role of different early-life elements for the consequences of early-life adversity.** Early-life adversities in the form of early-life stress, under/malnutrition and infection are known to modulate hippocampal development and altogether determine hippocampal structure and function in adulthood with adverse effects on learning and memory. During the early sensitive period of development the offspring is fully dependent on the mother. Maternal care encompasses several elements (sensory stimuli, transfer of nutrition, hormones, and antibodies). In fact it is mostly via disruption of maternal care (with exception of early-life infection which can directly act upon the offspring) that early adversities will elicit disruptions in hormonal, (neuro)inflammatory and nutritional profiles in the offspring. Because these elements affect one another, they will ultimately act synergistically to modulate hippocampal structure and function throughout life.

tight interaction and possible synergistic effects of stress and nutrition on neurocognitive development has been recently reviewed and discussed both prenatally (Monk et al., 2013) as well as postnatally (Lucassen et al., 2013), we will here focus on the interaction of early-life stress and malnutrition with the immune activation.

## WHAT IS THE EVIDENCE FOR AN INTERACTION BETWEEN EARLY-LIFE IMMUNE ACTIVATION AND EARLY-LIFE ADVERSITY?

Early-life adversities like stress and malnutrition not only lead to the previously described effects on cognitive and hippocampal function but also to changes in adult immunological function. The stress and immune systems have a strong interactive profile, illustrated by, e.g., the immune-suppressive effect of corticosteroids (Tsigos and Chrousos, 2002; Chaplin, 2010). Evidence of this relation in early-life has been provided by the enhanced pro-inflammatory status, both basally as well as in response to stress (Carpenter et al., 2010; Chen et al., 2010), of adult individuals with a history of early-life adversity, such as children raised in poor socioeconomic status households or who suffered from childhood maltreatment. This association has been confirmed by preclinical studies as well. In general, early-life stress paradigms (pre- and postnatal) lead to an immediate immunosuppressive state, e.g., a reduced lymphoproliferative response of the thymus (Llorente et al., 2007), downregulation of anti-inflammatory IL-10 and pro-inflammatory IL-1β (Dimatelis et al., 2012) and reduction of lipobinding protein (LBP), that regulates innate immune system pathogen presentation by microglia cells (Wei et al., 2012). These alterations in the immunoreactive profile in early-life are further accompanied by central changes in microglia morphology and number of Iba1 (Diz-Chaves et al., 2012) or lectin immunoreactive cells (Gómez-González and Escobar, 2009b).

In adulthood, the programming effect of early-life adversity by maternal deprivation on immune regulation is further illustrated by enhanced IL-1β responsiveness due to elevated IL-1 receptor levels at the post-synapse of adult hippocampal neurons (Viviani et al., 2013). In addition, the inflammatory response to an inflammatory challenge (systemic LPS injection) in the hippocampus of prenatally stressed mice is exaggerated in the reactivity of microglia and expression of pro-inflammatory cytokines (Diz-Chaves et al., 2013). Altogether, early-life stress tends to have a programming effect on neuroimmune functions, mainly resulting in an immediate immunosuppressive, but pro-inflammatory state in adulthood, which triggers an exaggerated neuroimmune response defined by cytokine secretion and microglia activity upon an immune challenge. How the early-life adversity-induced pro-inflammatory adult profile in the brain interacts with the other changes in brain structure and how these altogether lead to the observed cognitive impairments needs to be further investigated.

Next to the evident programming effects of early-life stress on neuroimmune functions, possible lasting effects of inflammatory challenges during early-life on HPA axis activity need to be considered as well. Early-life infection generally leads to a direct elevation of circulating glucocorticoids in early-life (Bilbo et al., 2005a) and while basal CORT was not affect by early-life infection at P14, the level of phosphorylated GR is significantly higher in the prefrontal cortex, but not the hippocampus (Dinel et al., 2014). In

contrast, early infection does not affect basal and/or stress-induced CORT in a lasting manner (Bilbo et al., 2006; Walker et al., 2010; Babri et al., 2014a; Dinel et al., 2014). In line with this, CORT secretion following adult LPS exposure seems independent of the early-life history of exposure to stress or infection (Bilbo et al., 2005a, 2007; Kohman et al., 2008). However there is evidence indicating that early-life infected animals exhibit prolonged CORT elevations accompanied by a greater content of pro-inflammatory IL-1β and TNFα in the hippocampus upon adult stress exposure (Walker et al., 2010) while exposure to high doses of the pro-inflammatory cytokine TNFα increased stress-induced CORT release (Babri et al., 2014b).

Clearly, in the activation of neuroimmune cells induced either by a peripheral immune challenge or by early-life adversity, the BBB plays a pivotal role. There is evidence that development of the BBB is hampered after exposure to perinatal stress and exposure to an early-life immune challenge, revealing elevated BBB leakage in among other areas the hippocampus (Gómez-González and Escobar, 2009a). Whether these changes in early-life stress induced BBB leakage are related to changes in neuroimmune functioning after early-life stress remains to be determined.

## WHAT IS THE EVIDENCE FOR AN INTERACTION BETWEEN EARLY-LIFE MALNUTRITION AND NEUROIMMUNE ACTIVATION?

Next to early-life stress, also early-life nutritional insults can affect the neuroimmune system. For example, there are indications for a strong association between circulating leptin levels and the suppression of lymphoproliferative responses and pro-inflammatory cytokine secretion in protein malnourished infants, both before and after recovery following refeeding (Palacio et al., 2002).

Similar indications are provided by preclinical studies where adult offspring of food-restriction dams have increased basal immune activity (measured as C-reactive protein) in female offspring at 9 months of age, but reduced cytokine induction (IL-1β and IL-6 secretion) in response to a second immune insult with LPS (Desai et al., 2009). Similarly, adult offspring of dams that were protein-deprived during lactation show an impaired responsiveness to a peripheral immune challenge, that was accompanied by elevated levels of basal and response CORT (Barja-Fidalgo et al., 2003). Lipid content of the diet early in life seems to be a strong modulator of neuroimmune functioning throughout life. Indeed, offspring of dams fed high-fat and high-trans fat during pregnancy and lactation exhibit increased basal immune activity (C-reactive protein) at birth and increased active microglia in adult (Bilbo and Tsang, 2010) associated with improved performance in the MWM. These basal changes are accompanied by an exaggerated peripheral and hippocampal IL-1β response to adult LPS (Bilbo and Tsang, 2010), classically known to activate microglia (Van Dam et al., 1992). Interestingly, when maternally deprived rats (P9) are weaned on a high fat diet during adult life, they present an increased pro-inflammatory modulation of IL-1β and TNFα in the hypothalamus (Mela et al., 2012). Omega-3 FAs in particular appear responsible for these observations as they activate neuroprotective signaling pathways (Calon and Cole, 2007) and act upon immune regulators, by, e.g., blockage of the NFκB signaling pathway (Singer et al., 2008). In fact, male offspring of omega-3 deficient dams exhibit a promotion of reactive inflammatory microglia and elevated pro-inflammatory cytokines in the hippocampus at P21 (Madore et al., 2014). It remains elusive how the changes in dietary fat composition early in life and subsequent priming of microglia further relate to levels of adult hippocampal neurogenesis.

One of the possible mediators of the interaction between nutritional intake and immune system is leptin, which is secreted by white adipose tissue (Fernández-Riejos et al., 2010). In fact, rats treated with LPS at P10 have increased food intake in adulthood associated with elevated circulating leptin levels. A second immune challenge (LPS at 7–8 weeks of age), while leading to an elevation in leptin serum level in animals that were never exposed to infection before, did not alter leptin levels in the neonatally infected animals (Iwasa et al., 2010). Moreover, neonatal overfeeding, similarly to the combination of early-life stress and a high fat diet as discussed above (Mela et al., 2012), leads to microgliosis in the hypothalamic regions, including the PVN of the hypothalamus, a key nucleus in the regulation of HPA axis activity that can be triggered by interleukin-1 as well (Berkenbosch et al., 1987). Especially in this region, microglia activation is overly exaggerate upon an immune challenge with LPS in adulthood (Ziko et al., 2014). Interestingly, these manipulations lead to a disruption in the patterns of leptin, coinciding with the leptin surge for normal hypothalamic development (Ahima et al., 1998; Ahima and Hileman, 2000). Thus, a disrupted pattern of leptin secretion and induced (neuro) inflammation by these manipulations may play an important role in programming of the cognitive functions (Miller and Spencer, 2014). It is remarkable that entirely different manipulations as prenatal stress (Diz-Chaves et al., 2013), being raised by a dam exposed to high fat diet during pregnancy and lactation (Bilbo and Tsang, 2010) and neonatal overfeeding (Ziko et al., 2014) reset the neuroimmune function and lead to exaggerated microgliosis in response to a subsequent immune challenge in adulthood.

Although not extensively studied during early-life, current evidence on the dietary supply of methyl donors modulating the present levels of homocysteine in the (developing) brain (Blaise et al., 2007; Troen et al., 2008), with a deficiency leading to hyperhomocysteinemia, further suggests a role for dietary methyl donors in microglia properties and activity in adulthood. Hyperhomocysteinemia is associated with an elevated levels of homocystein-presenting apoptotic cells, and also with enhanced proliferation of microglia in the brain (Zou et al., 2010). Moreover, hyper-homocysteinemia can be a risk factor or marker of neurodegenerative disorders in which cognitive dysfunction and neuroimmune functioning play an important role (Morris, 2003; Van Dam and Van Gool, 2009). However, to date, the exact relation of early-life methyl donor supply and neuroimmune functions remains elusive.

Exciting new evidence further supports a strong interaction between nutrition and immune system in the programming of hippocampal structure and function. A recent paper by Liu et al. (2014) proposed a pathway of "lactocrine" programming of hippocampal development and function by maternal deficiency of TNFα, resulting in altered chemokine composition of the mother. In fact, TNFα deficiency in mothers milk lead to impaired hippocampal proliferation and spatial memory in the offspring of

these animals, a clear indication of programming via nutritionally provided immune effector messengers (Liu et al., 2014). The exact pathway of TNFα and the role of chemokines that lead to alterations in hippocampal development, adult learning and memory, remains to be explored (Parylak et al., 2014).

Altogether, dietary composition during critical sensitive periods of development seem to be strongly involved in the immediate and lasting effects on the innate immune system, with a tendency to an immediate immunosuppressive response being associated with protein and fat malnourishment, and an enhanced pro-inflammatory activity induced by high fat diets associated with an sub-optimal neuroimmune response (too little or exaggerated) in response to later life immune challenges. How the early-life nutrition directly programs neuroimmune function and interacts with the neuroendocrine system, and programs cognitive functions and hippocampal neurogenesis in later life requires further study.

In the previous section we have highlighted some of the key elements of the early-life environment that might play an important role in the programming of cognitive functions by early-life adversity. As evident from the studies that we discussed these elements clearly do not act alone but rather in a synergistic manner. We discussed some of the possible mechanisms that could mediate the effects of early-life stress, malnutrition, and infection and discussed the evidence for their interactive profile. However clearly our discussion is not exhaustive and other equally important paths and mediators responsible for the final programming effect could be considered. For example, next to leptin, ghrelin a pancreatic hormone released upon hunger can influence not only eating behavior but stress, immune function as well as cognition (Diz-Chaves, 2011). Clearly having to consider so many different elements simultaneously renders the picture very complex and questions which are the best systems to target to prevent and/or reverse the deleterious effects of early-life adversity. In the following section, we will discuss some of the intervention strategies that have been explored up to date.

## EARLY-LIFE ADVERSITY; OPPORTUNITIES FOR INTERVENTION LATER IN LIFE

Adversities in the early-life period provoke thus immediate and programmed effects on different levels with lasting consequences for hippocampal function. Identification of these consequences and the different systems at play during early-life is essential to design optimal intervention studies to counteract the more complete set of consequences following early-life adversity. In recent years, multiple intervention studies have been performed to counteract either the lack of nutritional components, the consequences of early-life stress or the pro-inflammatory state after early-life infections. For instance, clinical research revealed the potential of high levels of maternal warmth (regarded as a positive experience) to overcome the programmed effects of the aversive low socioeconomic status on the immune system during early-life (Chen et al., 2010). However, considering the evidence presented in this review that these systems interact and affect each other so tightly and that they might thus act synergistically to program brain structure and function for life, the question arises as to which consequences of early-life adversity to target and whether there is a crucial time window for these interventions for optimal beneficial effects of these interventions. Here, we will discuss a few examples of potential intervention studies.

Because changes of HPA axis modulators are suggested as potential regulators of the lasting changes following early-life stress, suppression of these modulators has been investigated as a possible intervention in later life. For example, selective blockage of CRF receptor 1 immediately after the first week after chronic early-life stress exposure from postnatal day 10–17 in rats prevents hippocampal impairments in cognitive functioning and long-term potentiation (Ivy et al., 2010).

Enriching the later life environment, a manipulation that is known to stimulate hippocampal neurogenesis and improve performance of hippocampus related spatial behavioral tasks in adulthood (Kempermann et al., 1997; Nilsson et al., 1999), has been explored as well. For example, housing maternally separated rats in an enriched environmental condition during adulthood reversed the early-life stress induced changes in hippocampal GR and CRF expression (Francis et al., 2002). These manipulations do not only modify HPA axis activity but also affect neuroimmune functioning and activity of glial cells (Olah et al., 2009; Williamson et al., 2012; Gebara et al., 2013). In addition, there is evidence that enriching early-life environment by artificially increasing sensory stimuli by the mother (via handling) interferes with the adult pro-inflammatory programming of early-life *E. coli* infection (Bilbo et al., 2007). The adult LPS-induced increase of microglia (CD11b) and astrocyte (GFAP) markers and IL-1β levels in the blood and different brain regions of animals with a history of *E. coli* exposure was fully prevented by early-life handling. These data clearly suggest a strong interaction between sensory stimuli and infection early in life in the programming of the adult neuroimmune system (Bilbo et al., 2007).

A final manner to intervene with the consequences of early-life stress is modulation at the level of the epigenome. Early-life stress and early-life nutrition program later life function through alterations in chromatin structure and gene expression as became evident from clinical and animal studies. There is indeed increasing evidence that epigenetic mechanisms might be responsible for the early-life adversity induced life-long alterations in gene expression. (Heijmans et al., 2008; Murgatroyd et al., 2009; Steegers-Theunissen et al., 2009; Canani et al., 2011; Chen et al., 2012; Lucassen et al., 2013). Moreover, epigenetics mechanisms play a role in neuroinflammatory responses as well (Garden, 2013). Which are the factors regulating epigenetic mechanisms is yet unclear, however, there is growing interest in the role that nutrition might play in this context (Lucassen et al., 2013; Spencer, 2013). Nutritional interventions to prevent or reverse these epigenetic alterations have been only explored concerning metabolic programming but might certainly have the potential to intervene with the deleterious programming by early-life adversity of brain structure and function (Lucassen et al., 2013). For instance, folic acid supplementation to the offspring of protein-restricted diet fed dams during adolescence altered the protein-restricted metabolic outcome and modified the epigenetic alterations (Burdge et al., 2009). In addition, folate deficiency of the maternal diet during gestation negatively influences hippocampal developmental neurogenesis, but supplementation with the interrelated methyl donor

choline modified some of these effects on the neural progenitor cells (Craciunescu et al., 2010). But can dietary intervention later in life prevent or reverse the early-life adversity induced phenotype? Weaver et al. (2005) provided evidence that the programming effect of maternal care during early-life on the epigenetic modifications of the GR remain sensitive to alterations in adulthood, as central infusion with L-methionine could reverse the programmed effects of maternal care.

Overall, these studies indicate that environmental, nutritional, and pharmacological interventions, either during early-life or in adulthood, have the potential to modulate one or more consequences of early-life adversity. Currently, intervention studies lack some depth on the interplay of stress mediators, neuroimmune activity and the nutritional profile in how they might synergistically modulate hippocampal structure and function. Addressing the complexity of the early-life environment at large, rather than focusing on a single element will provide the necessary information to design new interventions, or a combination of interventions, that may fully prevent and/or reverse the consequences of early-life adversity.

## FINAL CONCLUSION

Well-known factors such as genetic vulnerability, gender, life style, and aging contribute to disorder vulnerability. In addition, early-life adversity further determines brain susceptibility to develop adult-onset psychopathologies and cognitive impairments later in life. Multiple elements (including stress, nutrition, and infections) in the early-life environment are crucial for proper hippocampal development, and structure and function in adulthood. Thus there is growing evidence that disruption of either of these elements has detrimental effects on cognitive functions, hippocampal structure, neurogenesis and the activity of neuroimmune cells in the hippocampus. Here, we have focused on how these different elements might interplay during early-life adversity and elicit similar effects on hippocampal neurogenesis and cognition in adulthood. Even though the interplay of these three elements is generally not considered in depth, the ultimate consequences are probably a synergistic effect and combination of these elements. Considering the intense cross talk between these elements and how they, together, program hippocampal structure and function, will provide important insights and contribute to novel targets for pharmacological, nutritional or life style interventions after early-life adversity.

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# Vitamin D and hippocampal development-the story so far

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Epidemiological studies suggest that vitamin D insufficiency may be prevalent in young as well as older populations. The pleiotropic effects of vitamin D are now beyond dispute and a growing number of studies provide accumulating evidence of a role for vitamin D in brain development and function. A number of studies to date have investigated the effects of early-life vitamin D deprivation on adult hippocampus in animals and humans, and there is a growing body of evidence to suggest a role for this hormone in the development of selected hippocampal functions such as latent inhibition and hole board habituation in rats. There are few studies to date of vitamin D deprivation or supplementation on early hippocampal development *in vivo*. However, a small number of studies, mostly *in vitro*, point to a role for vitamin D in differentiation and development of hippocampal neurons. There is also limited evidence that supplementation with vitamin D following a period of deprivation is capable of restoring cellular activity and later function. Further avenues of future research are outlined including animal studies on the effects of vitamin D deprivation and inadequacy on early hippocampal biochemistry and function, e.g., measurement of BDNF levels, GABAergic activity, long-term potentiation (LTP) and spatial navigation. It also remains to be established if there are critical developmental windows during which vitamin D is required. In light of the importance of the hippocampus in LTP and spatial learning, further investigations on the early effects of vitamin D deprivation on hippocampal development are warranted.

**Keywords: vitamin D, hippocampus, neurogenesis, differentiation, proliferation, LTP, depletion, repletion**

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## Introduction

Until a little over 30 years ago, the role of hormonal vitamin D was classically associated with calcium homeostasis, bone formation and maintenance, its up-regulation by parathyroid hormone being known for many years. However in recent years, myriad other functions and roles for vitamin D have been gradually postulated and verified, and its pleiotropic effects are now beyond dispute (Kalueff and Tuohimaa, 2007; McCann and Ames, 2008; Lai and Fang, 2013; Schlogl and Holick, 2014). Some examples of its diverse range of effects include immune-modulatory and pro-differentiation activity, cellular regulation and apoptosis, anti-inflammatory and antimicrobial activity, insulin secretion, interaction with the renin-angiotensin system, neuroprotection, *inter alia*. (See for examples, DeLuca and Cantorna, 2001; Garcion et al., 2002; Holick, 2003; Lin and White, 2004; Nagpal et al., 2005; Norman, 2006). Vitamin D is now known to be synthesized in many cells such as skin, lymph nodes, colon, adrenal medulla and pancreas (see for example, Zehnder et al., 2001; Lai and Fang, 2013) and it regulates approximately 3% of the human genes via its endocrine effects (Bouillon et al., 2008).

Among the general population, pregnant women are considered to be at risk of vitamin D deficiency (VD). The increased demands of the developing fetus combined with a possible decrease in environmental exposure to sunlight, may result in diminished circulating vitamin D (Hillman and Haddad, 1976; Markestad et al., 1983). One large US study reported that 12% of women aged 25–29 had low serum 25-hydroxyvitamin D levels (Looker and Gunter, 1998) while another study of young Canadian women reported even higher prevalences (Vieth et al., 2001). A high prevalence of VD has also been reported in infants, children and adolescents from various countries (Huh and Gordon, 2008). In addition, women who wear concealed clothes are also at risk and in one study, hypovitaminosis was observed in 82.5% of such a cohort (Belaid et al., 2008). The question of reduced vitamin D concentrations on consequent neonatal, infant and child development in relation to the hippocampus is therefore highly pertinent.

## Vitamin D and the CNS

The earliest evidence for binding of Vitamin D3 within the nervous system was first obtained over 35 years ago by the seminal autoradiographic studies of Stumpf et al. (1979, 1980), who reported its accumulation in the posterior pituitary, forebrain, hindbrain and spinal cord. However an earlier study by Clemens et al had reported that antibody to the Vitamin D receptor (VDR) within the dorsal hippocampus was strikingly similar to the D3 receptor (Clemens et al., 1985). Further autoradiographic studies by Stumpf and O'Brien (1987) demonstrated the positive presence of nuclear Vitamin D staining in many other brain regions including the ventral hippocampus. On the basis of the extensive presence of vitamin D within the brain, they proposed that calcitriol be renamed solitriol, the sunlight activated steroid hormone, to reflect its wider physiological remit. Since then the investigation of a neural role for vitamin D has gained significant traction and a plethora of animal studies have shed light both on its presence and its biological activity within the CNS independent of transport via the blood-brain barrier. For example, a number of studies have revealed the expression of VDR in specific brain regions including the thalamus, cerebellum, amygdala, cingulated gyri, temporal lobe, cerebral cortex, and hippocampus (Clemens et al., 1988; Prüfer et al., 1999; Langub et al., 2001; Zehnder et al., 2001; Garcion et al., 2002; Eyles et al., 2003; McGrath et al., 2004). Biosynthetic and degradative enzymes for the vitamin were also reported in glial and neuronal cells *in vitro* (Clemens et al., 1988; Neveu et al., 1994a,b; Baas et al., 2000). An important study by Eyles et al established for the first time that the VDR and α-1 hydoxlyase enzyme co-localized in specific areas of the brain (Eyles et al., 2005). This was followed by several functional studies on physiological and cellular effects of vitamin D on various aspects of brain cellular function (for examples see Ko et al., 2004; Lin et al., 2005; Taniura et al., 2006).

A growing body of evidence also points to a role for vitamin D in the development of the CNS. Vitamin D3 receptors have been located in the CNS of the rat embryo (Veenstra et al., 1998). In addition, transient deprivation of vitamin D early in life leads to changes in the new-born rat brain which may persist into adulthood. The offspring of vitamin D deficient rats have larger lateral ventricles (Eyles et al., 2003; Féron et al., 2005), a thinner cortex (Eyles et al., 2003) diminished levels of nerve growth factor (NGF; Eyles et al., 2003; Féron et al., 2005), and reduced expression of a number of genes involved in neuronal structure and neurotransmission (Féron et al., 2005). These changes do not persist if young animals are fed a vitamin D replete diet at birth but persist if they remain on a vitamin D deficient diet for the first 3 weeks of life (Féron et al., 2005). VD has also been shown to interfere with cortical development in developing rat brains by inducing fewer apoptotic cells at birth and more mitotic cells overall (Ko et al., 2004). Targeted gene arrays specific for apoptosis and cell cycle genes confirm a pattern of transcription deregulation in the deplete group consistent with the known properties of vitamin D (Ko et al., 2004). The latter study suggests that the known pro-apoptotic and pro-differentiating properties of Vitamin D may also play a role in brain development. In addition, vitamin D has been demonstrated to increase levels of NGF and neurotrophins NT-3 and NT-4 in cultured glial cells, astrocytes and oligodendrocytes (Neveu et al., 1994a,b; Baas et al., 2000). Neurotrophins (NT) play an important role in the survival of developing neurons and in the proliferation and differentiation of neural progenitor cells (Davies, 2004). Its overall contribution to healthy brain development renders also likely a role for vitamin D in the developing hippocampus. A recent paper investigated the effects of calcitriol on neural stem cell differentiation from cultured mouse neurospheres, and reported concentration-dependent increases in the numbers of cells containing the intracellular neural marker NeuN, and the oligodendrocyte marker GalC, and the astrocyte marker GFAP. They also reported increased VDR expression within the neural stem cells (Shirazi et al., 2015).

## Studies on the Hippocampus

Functional VDRs were first reported in the dentate gyrus (DG), pyramidal and granule layers, glial cells and in subfields CA1–3 of the rat hippocampus, which were capable of specifically binding DNA response elements to osteopontin (Langub et al., 2001). Following the discovery of VDR within the hippocampus, a number of studies have investigated the effects of pre or perinatal VD or supplementation on aspects of hippocampal structure or function in rodents. Several studies have also been carried out on cultured hippocampal cells *in vitro*. Although many of the *in vivo* studies have been conducted on brains of adult rodents, changes in structure or function in adult brains provide circumstantial evidence for impaired development, as reported deficits represent the outcomes of earlier anatomical or physiological abnormalities extending into adulthood. Therefore they are germane to an overall understanding of the role of vitamin D in hippocampal development.

## Behavioral Studies

Several studies have investigated the effects of prenatal VD deficiency in rodents on cognitive and behavioral functions which directly or indirectly involve the hippocampus. In one animal model, offspring of female rats who had been subjected to a VD deficient diet from 6 weeks prior to mating until the birth of the litter were evaluated for behavioral changes at various time points following repletion of Vitamin D at birth. Significant impairment of both latent inhibition and hole-board habituation in vitamin-deprived rats was reported (Becker et al., 2005). The former is considered to be a measure of the ability to learn to ignore irrelevant stimuli. The latter is a form of non-associative learning in which there is a progressive diminution with repetition of a specific stimulus and also represents the ability to ignore irrelevant stimuli. Both functions are thought to constitute a central feature of schizophrenia (Becker et al., 2005) and involve the hippocampus (Oades and Isaacson, 1978; Weiner, 1990). However there was no impairment of spatial learning within a radial maze, nor on two-way active avoidance learning. Vitamin depleted rats also demonstrated superior ability to maintain previously learned rules in a brightness discrimination task. The authors concluded that exposure to low prenatal vitamin D has a selective impact on certain aspects of memory only, with disruption of latent inhibition, but no effect on memory acquisition and memory retrieval. The apparently superior ability of VD rats to maintain previously learned rules in a brightness discrimination task is an intriguing finding which needs to be replicated. However this link at present remains speculative, albeit tenable. Apropos its functional significance, it is tempting to speculate that it may constitute a compensatory physiological mechanism in the absence of sufficient sunlight. A later study by the same group investigated synaptic plasticity and long-term potentiation (LTP) in the DG of the hippocampus (Grecksch et al., 2009). Surprisingly, VD induced an enhancement of LTP using weak or strong titanic stimulation which persisted for more than 24 h. The authors concluded that memory acquisition and retrieval was unaffected and only latent inhibition disrupted. They also noted that the finding of enhanced LTP was in good agreement with the improved memory associated with the brightness discrimination task of their earlier study. Harms et al investigated the effect of prenatal VD on different neurological behaviors in two strains of 10 week old mice, and noted increased frequency of head-dipping in the hole board test in both strains, indicative of increased exploration and hippocampal involvement (Harms et al., 2012). The clinical significance of the latter has not been evaluated to date. In a separate study by the same group, one strain of mouse displayed spontaneous hyperlocomotion, pointing to intraspecies as well as inter-species differences in responses to developmental VD (Harms et al., 2008).

In light of the major role of the hippocampus in long-term memory formation, the apparent lack of effect of VD on spatial learning and memory formation and retrieval is an important finding which needs to be verified across species. An investigation of BDNF levels in such studies would also be useful, given its importance in spatial learning and hippocampal plasticity (Kang and Schuman, 1995a,b; Leal et al., 2015). It must be borne in mind also, that rats in the cited studies received some vitamin D from their mothers from the time of birth until weaning, and it is possible that this could have resulted in a partial restoration of hippocampal structure and function.

The hippocampus constitutes part of the neural circuitry responsible for the acoustic startle reflex (Swerdlow et al., 2001). One study to date has investigated the effects of vitamin D deprivation over time on this reflex in rats (Burne et al., 2004). Several groups of rats were treated and compared e.g., no depletion, replete at birth, replete at weaning, depleted until 10 weeks of age, or depleted between 5 and 10 weeks of age, and they found that only the combined prenatal and chronic postnatal vitamin D deprivation, but not early life hypovitaminosis on its own, resulted in an impaired response. This suggests that early VD for a limited period does not automatically produce any long-term adverse effects on brain function.

## Neuroanatomical and Neurochemical Studies

Harms et al investigated the effect of prenatal VD on brain anatomy in two strains of male and female new-born mice and found a significantly reduced hippocampal volume in females but not males. However the phenotype did not extend into adulthood, suggesting that either normal postnatal development or the reintroduction of vitamin D at birth may have corrected the deficit (Harms et al., 2012). The finding of reduced hippocampal volume in females is noteworthy and in need of further replication. Its clinical significance if any, is presently unknown and can only be elucidated in the context of further specific behavioral studies comparing hippocampal function and activity in females and males. One other study on the offspring of VD mice has demonstrated smaller lateral ventricles at 30 weeks but normal hippocampal volume (Fernandes de Abreu et al., 2010). There are no studies to date on the effects of VD on hippocampal volume in rats or other species.

Two animal studies to date have investigated the effect of VD on hippocampal neurogenesis. The first study, utilising a 1-α hydroxylase knock-out mouse model investigated the hippocampus of 8 week old mice born of vitamin D-depleted dams following injection with labeled bromodeoxyuridine (bdU; Zhu et al., 2012). They found a 50% reduction in the number of what were referred to as newborn neurons within the subgranular zone of the DG, alongside an increase in the number of apoptotic cells, 7 and 28 days post-injection. Subsequent dietary supplementation with vitamin $D_3$ was able to prevent the reduction in bdU-labeled DG cells. This was paralleled by a decrease in NGF levels within the brain, leading the authors to postulate on a direct link between both phenomena. The decrease in NGF levels is consistent with previous studies in relation to brain development (Eyles et al., 2003; Féron et al., 2005). However the finding of increased numbers of apoptotic cells in the hippocampus is in contrast to the

findings of decreased numbers of apoptotic cells at birth in the developing cortex (Ko et al., 2004) and with the known overall effects of vitamin D on apoptosis. A second study used rats to investigate the effect of gestational VD on their 10 week old offspring and also reported reduced neuronal proliferation within the subgranular layer of the DG (Keilhoff et al., 2010).

It appears that the negative effects of early transient VD on the hippocampus can be counteracted, under certain conditions at least, by subsequent repletion *in vivo* (Eyles et al., 2003; Burne et al., 2004; Féron et al., 2005; Harms et al., 2012; Zhu et al., 2012). This suggests that VD under certain conditions, exerts only temporary rather than long-term deficits in structure and function. It remains to be established if this compensatory ability is limited to specific ontogenetic windows of development. More carefully differentiated investigations like this one on various aspects of hippocampal development and function are necessary.

Two studies have investigated the effect of a single treatment of 50 μg of vitamin D on neurotransmitter concentrations in offspring of rats, and found that within the hippocampus, levels of serotonin and 5H1AA, but not dopamine or HVA, were negatively affected by the single treatment, which the authors referred to as hormonal imprinting (Tekes et al., 2009a,b). This is an interesting finding which warrants further exploration over longer periods with repeated hormone treatments. Apart from elucidating valuable data on the direct effect of vitamin D on neurotransmitter levels within the hippocampus, such studies carried out on young animals could also shed valuable light on hippocampal development when combined with behavioral testing. A recent study investigated calcitriol supplementation for 6 weeks in rats and reported higher GABA levels in the hippocampus and cortex (Jiang et al., 2014).

## *In Vitro* Studies

Several groups have investigated vitamin D supplementation of up to 100 nM on developing cultured hippocampal neurons (Brann et al., 1999; Brown et al., 2003; Marini et al., 2010). Two of these reported arrested or reduced mitosis and cell division along with accelerated neurite outgrowth and increased NGF production (Brann et al., 1999; Brown et al., 2003). These findings are consistent with an earlier study demonstrating the upregulation of the anti-mitotic cyclin-dependent kinase regulators p. 21 and p. 27 in a related cell line (Rots et al., 1999). Marini's group reported translocation of vitamin D from the cytoplasm to the nucleus and back to the cytoplasm, which caused a delay in cell proliferation and induction of cell differentiation (Marini et al., 2010). They expanded their study to include a simultaneous investigation of cell cycle activity during Vitamin D supplementation. They found reduced expression of two proteins involved with proliferation (PCNA and cyclin D1) between 12–15 h post incubation compared to controls. This coincided with a 70% decrease in thymidine incorporation into DNA and with the G1/S phase of the cell cycle. There was a concurrent 16-fold increase in levels of Bcl2, a protein marker of cell differentiation, and NF-L, one of the neurofilaments involved in maintenance and remodeling of the neuronal cytoskeleton. They also reported an increase in the development of dendrites and axons over 5 days of culture compared to controls. In a later study, the same group found that when serum is withdrawn from cultured hippocampal cells, treatment with 100 nM vitamin D fails to trigger differentiation. However, increasing the dose to 400 nM allowed the interaction of the vitamin with its receptor, resulting in differentiation (Bartoccini et al., 2011). This finding points to differential effects of varying concentrations of vitamin D on cellular hippocampal development and warrant further exploration. Although the concentration of hormone used to supplement *in vitro* is higher than that detected in standard rat chow, vitamin D levels in humans have been found in more than one study to be elevated in pregnancy (Seki et al., 1991; Ardawi et al., 1997). It is therefore possible that local gradients of the hormone occur in the developing embryonic tissues and organs *in vivo* which may mirror if not exceed those used experimentally.

Cui et al examined the effect of VD on neuroprogenitor formation in cultured neonatal rat brain cells (Cui et al., 2007). They found an increase in the number of neurospheres formed in culture from the subventricular zone (SVZ), which was unaffected by subsequent supplementation with vitamin D. The authors concluded that vitamin D can regulate cellular proliferation in the developing brain. It is noteworthy that exogenous vitamin D supplementation of an initially deprived medium failed to elicit any cellular response. This contrasts with the restoration of normal development following vitamin D repletion in whole animals (Féron et al., 2005), and highlights the potential disparity between *in vitro* and *in vivo* findings. It may reflect the lack of any pool of pre-existing progenitor cells in the *in vitro* studies compared with the *in vivo* situation. Interestingly, VDR positive staining was localized within the progenitor cells of control and deplete neonate brains, particularly within the SVZ, and was unaffected by vitamin depletion.

One *in vitro* study has investigated the interaction of glucocorticoids with vitamin D in isolated hippocampal cells (Obradovic et al., 2006). Pre-treatment with 1 μmol vitamin D for 24 h substantially reduced the degree of dexamethasone—induced apoptosis and induced the activation of the p42/p44 MAPK complex which is involved in cellular differentiation. Vitamin D also abrogated the inhibitory effects of dexamethasone on the MAPK complex. The antagonistic effects of vitamin D on glucocoticoid action was postulated to have potential significance in treatment of cognitive impairments and major depression, both of which are accompanied by high amounts of circulating corticosteroids (Holsboer, 2000).

## Future Directions and Conclusions

In light of the accumulating animal and *in vitro* evidence to date, it is likely that vitamin D plays a role in hippocampal development *in vivo*. However, the majority of animal studies to date have been carried on animals from several weeks old and upwards, and there are insufficient data on the effects

of VD on newborn and adolescent animals. This constitutes a major lacuna in our overall understanding of the role of vitamin D in the early development of the hippocampus and is an avenue ripe for exploration. There is a need for similar studies as those carried out to date on vitamin deficient new-born animals and continuing at regular timepoints to adulthood, to elucidate clearly how VD impacts on development *in vivo* both anatomically and structurally. Further anatomical studies are particularly necessary on early hippocampal neurogenesis and neuronal differentiation *in vivo* to confirm the *in vitro* findings.

It is possible that there are temporal variations in the effects of vitamin D such that particular time points along the spectrum of hippocampal development are more sensitive to its absence or its supply, than are others. There is a need for detailed studies investigating the effects of varying degrees of VD over time on hippocampal structure and function, given that VD in human subjects is invariably partial rather than total.

The overall behavioral findings to date with rodents on the effects of early VD on hippocampal function are complex and subtle, pointing to specific deficits such as latent inhibition and hole board habituation, rather than global disruption of function. These findings are also in need of replication to eliminate the possibility that some of these observations are species-specific rather than global. The finding, for example, of increased hyperlocomotion in one specific mouse species but not another (Harms et al., 2008) highlights the potentially differing effects of VD even within a single species.

Hippocampal structure and function is altered in schizophrenia, and the neuro-developmental hypothesis of schizophrenia suggests that an interaction between genetic and environmental factors during critical windows of development negatively impacts on brain development. Low prenatal vitamin D has been postulated as an epigenetic risk factor for same (McGrath, 1999), and transient VD is considered a developmental model in schizophrenia research. This has led to an important volume of work on this topic, some of which has been cited in this review in the context of the hippocampus. Hippocampal hyperactivity in schizophrenia has been demonstrated via neuroimaging and GABAergic mechanisms have been implicated in this effect (Heckers and Konradi, 2014). The recent finding of elevated GABA levels within the hippocampus and cortex following calcitriol supplementation in adult rats is noteworthy in this regard (Jiang et al., 2014). The effect of VD on neuronal activity and on GABA levels within the developing hippocampus *in vivo* is also worth investigating.

Given the known adverse effects of glucocorticoid excess on early brain development and the ensuing propensity to psychiatric illness later in life, another worthwhile avenue of research is the exploration of combined glucocorticoid excess with VD over varying time periods pre and post natally on hippocampal development and function *in vivo*. In particular, their combined effect on neural plasticity and LTP within the hippocampus needs to be elucidated.

It also remains to be established whether or not prenatal or perinatal VD may result in subtle changes in the developing hippocampus which render it more susceptible to eventual development of neurodegenerative diseases such as Alzheimer's and Parkinson's. While a number of studies have looked at the effects of VD on cognitive function in ageing or elderly populations, none to date have attempted to ascertain whether VD in early life could predispose to cognitive dysfunction in adulthood. Such studies of VD in the developing hippocampus should also measure BDNF levels, which are known to decrease in hippocampal neurons of Alzheimers patients (Siegel and Chauhan, 2000).

Finally, there are no cross-sectional studies to date in young children on hippocampal function such as memory formation and retrieval, and spatial navigation as a function of their vitamin D intake. This would shed the most valuable light possible on the role of vitamin D in early hippocampal development.

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MINI REVIEW
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# Early Life Stress Effects on Glucocorticoid—BDNF Interplay in the Hippocampus

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Early life stress (ELS) is implicated in the etiology of multiple psychiatric disorders. Important biological effects of ELS are manifested in stress-susceptible regions of the hippocampus and are partially mediated by long-term effects on glucocorticoid (GC) and/or neurotrophin signaling pathways. GC-signaling mediates the regulation of stress response to maintain homeostasis, while neurotrophin signaling plays a key role in neuronal outgrowth and is crucial for axonal guidance and synaptic integrity. The neurotrophin and GC-signaling pathways co-exist throughout the central nervous system (CNS), particularly in the hippocampus, which has high expression levels of glucocorticoid-receptors (GR) and mineralocorticoid-receptors (MR) as well as brain-derived neurotrophic factor (BDNF) and its receptor, tropomyosin-related kinase receptor B (TrkB). This review addresses the effects of ELS paradigms on GC- and BDNF-dependent mechanisms and their crosstalk in the hippocampus, including potential implications for the pathogenesis of common stress-related disorders.

**Keywords: early life stress, glucocorticoid, glucocorticoid receptor, BDNF, HPA-axis, TrkB, hippocampus**

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## INTRODUCTION

Glucocorticoids are steroid hormones and the end product of the hypothalamus-pituitary-adrenal (HPA) axis, which regulates the stress response. GC effects are mediated by MR and GR (De Kloet et al., 1998; McEwen, 1998; de Kloet et al., 2005a);. MR and GR are abundantly expressed in hippocampus and hippocampal function is implicated in both appraisal processes and stress adaptation. Through MR, GCs influence the brain's appraisal of novel information and memory retrieval, and thereby influence behavioral coping responses (de Kloet et al., 2005a). As GC concentrations increase in response to stressors, GR are activated to promote stress adaptation, reallocation of energy resources in preparation for future events and recovery of the system (de Kloet, 2003; de Kloet et al., 2005a).

One important target of GCs is BDNF-signaling, which is a crucial contributor to the modulation of axonal guidance, synaptic plasticity and neurite outgrowth (Jeanneteau and Chao, 2013). MR, GR and the BDNF receptor, TrkB, are co-expressed in hippocampal neurons,

supporting this region as the primary site of immediate interactions between the GC- and BDNF-signaling pathways (Jeanneteau et al., 2012).

In this mini-review we present the effects of ELS on GC- and BDNF-dependent mechanisms in the hippocampus using primarily evidence from ELS animal models. The most commonly used rodent and non-human primate model in this context is the maternal separation (MS) paradigms with each displaying variations in developmental age, repetition and duration (**Table 1**). GC- and BDNF-signaling pathways influence each other and here we propose that ELS provokes a change in their equilibrium that contributes to heightened risk for stress-related psychopathology.

## HPA-AXIS AND ELS

There is a distinct pattern of HPA-axis activity during early development first described in rodents, which maintains stable and low circulating GC levels during the stress hyporesponsive period [SHRP; postnatal days (pnd) 1–10 in mice and pnd 3–14 in rats] (Sapolsky and Meaney, 1986; de Kloet et al., 2005b). Early life experiences can disrupt the SHRP by elevating basal GC secretion and turning the HPA-axis responsive to subsequent stressors. Thus, ELS not only exerts acute effects but also impacts long-term developmental trajectories in the brain (de Kloet et al., 2005b). In humans, the SHRP occurs during the postnatal months 6–12 and adverse experiences during this period can have a long-lasting impact on the HPA-axis (Gunnar and Quevedo, 2007).

MS is an established procedure of inducing acute stress effects during early life that yields long-term effects. MS results in heightened HPA-axis responsiveness in the early postnatal period and triggers a variety of stress-related behavioral phenotypes in later life (Daskalakis et al., 2014). However, MS effects are dependent on many factors, including the duration and frequency of the separations, age of the pups, and context under which the pups experienced the separation from the dam (Rosenfeld et al., 1992; van Oers et al., 1998; Enthoven et al., 2010; Daskalakis et al., 2011). Furthermore, the long-term effects of MS depend on match or mismatch with later life context (Daskalakis et al., 2012). Studies using other ELS paradigms (variations of maternal care, limited nesting) demonstrate similar long-term effects that are mediated through GC-dependent mechanisms (Liu et al., 1997; Champagne et al., 2008; Ivy et al., 2008).

Long-lasting alterations in the HPA-axis induced by ELS in rodents have been linked to experience-dependent epigenetic modifications in regulatory regions of stress-related genes (Weaver et al., 2004; Murgatroyd et al., 2009). In humans, where early adversity is associated with adult stress-related disorders and HPA-axis dysregulation, similar epigenetic changes were reported as observed in the above-mentioned rodent studies (McGowan et al., 2009; Daskalakis and Yehuda, 2014; Ruby et al., 2015). Interestingly, epigenetic changes caused by ELS might depend on genetic predisposition (Klengel et al., 2013). Therefore, the interplay of genetic background (hit-1) with early experiences (hit-2), might create a vulnerable or resilient neuroendocrine profile which, upon adult stress exposure (hit-3), can produce an adaptive healthy or a maladaptive pathologic response (Daskalakis et al., 2013).

## BDNF SIGNALING AND ELS

ELS has consequences for structural and physiological properties of stress-sensitive brain regions and behavior. For instance, rats with a history of low maternal care displayed decreased hippocampal synaptogenesis, BDNF, long-term potentiation and memory at baseline (Liu et al., 2000). Neurotrophins are crucial mediators in the facilitation of brain connectivity, neuronal plasticity, synaptic integrity and the promotion of basal neurogenesis (Ghosh and Greenberg, 1995; Lee et al., 2002). The most abundant neurotrophin in the mammalian CNS is BDNF. It is synthesized in the endoplasmic reticulum as a pre-pro-molecule and undergoes two cleavage steps from pre-pro via pro-BDNF to its mature form, which is packaged in secretory vesicles (Pang et al., 2004; Revest et al., 2014). Upon neuronal activity, BDNF is released from the synapse and diffuses to its receptor TrkB. Upon BDNF-binding, TrkB undergoes homodimerization and autophosphorylation and thus, the activation of downstream signaling cascades involved in neuronal integrity and survival (Chao, 2003). Genetic modifications of BDNF have a crucial effect on synaptic plasticity as shown in an animal study using BDNF heterozygous (+/Met) for the Val66Met polymorphism. After 7 days of restraint stress with BDNF het and wild-type (WT) mice, BDNF het mice displayed reduced apical dendrite density in the prefrontal cortex (PFC) and in addition, impaired working memory in comparison to WT littermates (Yu et al., 2012). Moreover, BDNF influences synaptic transmission and its efficacy is influenced by this single nucleotide polymorphism (SNP) in its prodomain. In the same mouse model, the amount of NMDA receptor mediated currents in the hippocampus and the infralimbic medial PFC of BDNF Met/Met mice was significantly lower than in BDNF Val/Val mice (Ninan et al., 2010; Pattwell et al., 2012). The human BDNF gene seems to be under high selection pressure against genetic variability, since in a whole exome sequencing study of 14 schizophrenia trios and a subsequent study performing targeted exome capture in 48 sporadic schizophrenia cases, both cohorts displaying a high number of cases with childhood trauma, no novel genetic variants in the BDNF gene were observed (Kranz et al., 2015a,b).

BDNF-signaling is also influenced by ELS (Alleva and Francia, 2009). In humans, ELS can evoke significant memory impairments in adulthood (Bremner et al., 2003) in association with reduced BDNF levels (Grassi-Oliveira et al., 2008). Moreover, these associations depend on the Val66Met polymorphism (Chen et al., 2006; Elzinga et al., 2011; Molendijk et al., 2012). A similar pattern was observed for peripheral BDNF expression in young rhesus macaques. Carriers of the Met allele of the functionally ortholog polymorphism at codon 46 displayed decreased BDNF levels after maternal deprivation (Cirulli et al., 2011). Besides the combined effects of genetics and MS on BDNF expression, there are epigenetic effects associated with ELS. The relationship of MS and epigenetic

**TABLE 1 | Overview of rodent studies on BDNF signaling and Maternal Separation.**

| Paradigm | Species | *BDNF* gene regulation | BDNF protein | HPA-axis | Neurogenesis and synaptic plasticity | Behavior | References |
|---|---|---|---|---|---|---|---|
| MD<br>(pnd 3; 24 h once) | Brown Norway rats | Month 30–32<br>Basal: n.c. *BDNF* mRNA in HIP<br>+AS: ↓ *BDNF* mRNA in HIP (only in MD rats with MWM impairments) | | | | | Schaaf et al., 2001 |
| MD<br>(pnd 7; 3 h or 6 h once) | Sprague–Dawley rats | pnd 7; 3 h after onset<br>↑ *BDNF* exon III mRNA in HIP<br>6 h after onset<br>↓ *BDNF* exon I mRNA in HIP | | | | | Nair et al., 2007 |
| MD<br>(pnd 9; 24 h once) | Wistar rats | pnd 9; 2, 6 or 24 h after onset<br>n.c. *BDNF* mRNA in HIP<br>pnd 21<br>n.c. *BDNF* mRNA in HIP | | | pnd 21<br>n.c. *NMDA-R mRNA* in HIP | | Roceri et al., 2002 |
| | | pnd 72<br>↓ *BDNF* mRNA in HIP (but not further ↓ by AS) | pnd 72<br>↓ BDNF protein in HIP | | pnd 72<br>↓ *NMDA-R mRNA* in HIP | | |
| MD<br>(pnd 9; 24 h once) | Wistar rats | pnd 90—basal (males + females):<br>↓ *BDNF* mRNA in HIP<br>pnd 90 – CS (males + females):<br>↓ *BDNF* mRNA in HIP | | pnd 90—basal (males): ↓ GR in HIP<br>pnd 90 – CS (males): ↓ GR in HIP | | pnd 80–82<br>basal (males): ↓ NOR | Llorente et al., 2011 |
| MD<br>(pnd 9; 24 h once) | Wistar rats | pnd 98–112—basal:<br>↑ *BDNF exon I, IV* mRNA in dorsal HIP (males and females)<br>↑ *BDNF exon II, VII, IX* mRNA in dorsal HIP (males)<br>n.c. in ventral HIP (males and females)<br>↑ *BDNF I* mRNA in medial PFC (males)<br>↓ *BDNF VII and VIII* mRNA in medial PFC (males)<br>n.c. in CPu and NAc (males and females) | pnd 98–112—basal:<br>↓ BDNF protein in dorsal HIP (males) | | pnd 98–112—basal:<br>n.c. in CPu and NAc (males and females) | pnd 84–105—basal<br>basal: n.c. in short-term or long-term memory spatial memory, working memory, NOR, EPM | Choy et al., 2008; Hill et al., 2014a,b |
| | | pnd 98–112—CCORT:<br>↓ *BDNF* mRNA in HIP (males)<br>↑ *BDNF IX* mRNA and BDNF protein in medial PFC (males)<br>n.c. in CPu and NAc (males and females) | pnd 98–112—CCORT: ↓ BDNF protein in ventral HIP (females) | | pnd 98–112—CCORT:<br>↑ *DR3* mRNA in medial PFC (males)<br>↑ *DR2* mRNA in medial PFC (males)<br>n.c. in CPu and NAc (males and females) | pnd 84–105—CCORT: ↓ short-term spatial memory (males) and learning delay in long-term spatial (males) memory, ↓ sucrose preference (females)<br>n.c. working memory, NOR, EPM | |

*(Continued)*

**TABLE 1 | Continued**

| Paradigm | Species | *BDNF* gene regulation | BDNF protein | HPA-axis | Neurogenesis and synaptic plasticity | Behavior | References |
|---|---|---|---|---|---|---|---|
| MD (pnd 11; 24 h once) | Sprague–Dawley (mothers) and Long Evans (fathers) hybrid rats | pnd 11; 24 h after onset: ↓ *BDNF* mRNA in HIP | | 24 h after onset: n.c. ACTH, ↑ CORT | 24 h after onset: n.c. neurogenesis in HIP ↓ neurogenesis in parietal cortex | | Zhang et al., 2002 |
| RMS (pnd 1–14; 2 h daily) | C57BL/6J (B6) and Balb/cJ (Balb/c) mice | pnd 40 n.c. *BDNF* exon IV promoter methylation (males + females) in PFC and HIP n.c. *BDNF* exon IX promoter methylation (males + females) in PFC ↑ *BDNF* exon IX promoter methylation (males + females) in HIP n.c. *BDNF* exon IX promoter methylation (males + females) in PFC ↓ *BDNF* mRNA (females) in HIP ↓ *BDNF* mRNA (males + females) in PFC | | pnd 40 ↓ GR exon 1 methylation (females) in PFC ↑ GR exon 1 methylation (males) in HIP n.c. *GR* mRNA (males+females) in HIP n.c. *GR* mRNA (males + females) in PFC | | pnd 35 ↑ OF activity (males) pnd 35 ↓ sucrose preference pnd 40 ↓ social interaction (males) | Kundakovic et al., 2013 |
| RMS (pnd 1–14; 3 h daily) | Wistar rats | | pnd 104 ↓ BDNF protein in HIP (n.c. in PFC) | | | pnd 104 n.c. OF, n.c. NOR | Pinheiro et al., 2015 |
| RMS (pnd 1–21; 3 h daily) | Wistar rats | | pnd 56 ↓ BDNF protein in medial PFC n.c. BDNF protein in HIP n.c. BDNF protein in NAc | | | pnd 56 Partially impaired reversal learning performance | Xue et al., 2013 |
| RMS (pnd 2–6; 3 h daily) | Long-Evans rats | | pnd 7 MS prevented the conditioning associated ↓ BDNF protein in HIP ↓ BDNF protein in olfactory bulb | | | pnd 7 ↓ odor conditioning | Zimmerberg et al., 2009 |
| RMS (pnd 2–6; 5 h daily) | B6C3Fe reeler (C57/BLJ background) mice (wild-type presented) | | adult ↓ BDNF protein PFC and striatum (n.c. HIP) | | | adult ↓ social interaction (home cage activity n.c.) | Ognibene et al., 2008 |

*(Continued)*

**TABLE 1 | Continued**

| Paradigm | Species | *BDNF* gene regulation | BDNF protein | HPA-axis | Neurogenesis and synaptic plasticity | Behavior | References |
|---|---|---|---|---|---|---|---|
| RMS (pnd 2–14; 3 h daily) | Sprague–Dawley rats | pnd 17<br>basal: ↑ *BDNF* mRNA in HIP and PFC (+AS no further increase)<br>pnd 35<br>n.c.<br>pnd 90<br>↓ *BDNF* mRNA in PFC (+CS no further increase)<br>n.c. *BDNF* mRNA in PFC and striatum (+CS: prevented ↓ caused by CS) | | pnd 1<br>basal CORT: n.c.<br>+AS CORT: n.c.<br>pnd 13<br>basal CORT: n.c.<br>+AS CORT: n.c. | | | Roceri et al., 2004 |
| RMS (pnd 2–14; 3 h daily) | SERT knockout Wistar rats (wild-type presented) | pnd 85-95<br>n.c. *BDNF* mRNA in dorsal HIP<br>↓ *BDNF* total, 3′-UTR, exon IV mRNA in ventral HIP<br>n.c. *BDNF* mRNA in dorsomedial PFC<br>↓ *BDNF* total, 3′-UTR, exon IV mRNA in ventromedial PFC | | | | | Calabrese et al., 2015 |
| RMS (pnd 2–14; 3 h daily) | Sprague–Dawley rats | pnd 14<br>↑ *BDNF* exon II mRNA in HIP | | | pnd 15<br>n.c. neurogenesis in HIP SGZ | | Nair et al., 2007 |
| | | pnd 21<br>↑ *BDNF* exon IV and V mRNA in HIP | | | pnd 21<br>↑ neurogenesis in HIP SGZ | | |
| | | pnd 60<br>basal: n.c. *BDNF* mRNA<br>+AS: prevented ↓ in exon III, IV and V *BDNF* mRNA caused by AS<br>+ CS: prevented the increase in exon I and II and ↓ in exon III, IV and V *BDNF* mRNA caused by CS | | | | | |
| RMS (pnd 2–14; 3 h daily) | Sprague–Dawley rats | | pnd 68<br>+ JS: ↑ BDNF protein in ventral HIP (additionally MS prevented ↓ BDNF protein in dorsal HIP) | pnd 68<br>+ JS basal: ↓ ACTH and CORT | | pnd 67<br>+ JS: n.c. EPM and OF | Faure et al., 2006, 2007 |
| RMS (pnd 2–14; 3 h daily) | Sprague–Dawley rats | pnd 90<br>↑ *BDNF* mRNA in HIP | | | pnd 90<br>n.c. neurogenesis in HIP | | Greisen et al., 2005 |

*(Continued)*

**TABLE 1 | Continued**

| Paradigm | Species | *BDNF* gene regulation | BDNF protein | HPA-axis | Neurogenesis and synaptic plasticity | Behavior | References |
|---|---|---|---|---|---|---|---|
| RMS<br>(pnd 2–14; 3 h daily) | Long Evans rats | | pnd 95<br>↑ pro-BDNF in VTA<br>↓ BDNF protein in HIP, striatum<br>↑ BDNF protein in VTA | pnd 70–95<br>AS: ↑ ACTH and CORT | – | pnd 50–60<br>↑ locomotor activity, ambulation and grooming<br>↑ acoustic startle | Lippmann et al., 2007 |
| RMS<br>(pnd 2–14; 3 h daily) | Sprague–Dawley rats | | pnd 52<br>↓ BDNF protein in dorsal HIP and ventral HIP<br>pnd 101<br>↑ BDNF protein in ventral HIP | | pnd 101<br>n.c. MKP-1 levels in ventral HIP | pnd 65<br>n.c. OF activity<br>↑ 22 kHz vocalizations<br>↑ FST immobility<br>pnd 99<br>↓ EPM anxiety<br>pnd 100<br>↑ 22 kHz vocalizations<br>↑ FST immobility | Dimatelis et al., 2012, 2014 |
| RMS<br>(pnd 2–14; 3 h daily) | Sprague–Dawley rats | pnd 21<br>↓ H3K9 dimethylation in HIP<br>↑ *BDNF* exon IV mRNA in HIP<br>↑ *BDNF* mRNA in HIP | pnd 21<br>↑ BDNF protein in HIP | | pnd 21<br>↑ neurogenesis in HIP SGZ | | Suri et al., 2013 |
| | | pnd 60<br>↓ H3K9 dimethylation in HIP<br>↑ *BDNF* exon IV mRNA in HIP<br>↑ *BDNF* mRNA in HIP | pnd 60<br>↑ BDNF protein in HIP | | pnd 60<br>n.c. neurogenesis in HIP SGZ | pnd 60<br>↑ WWM escape latency<br>n.c. retention in WWM<br>n.c. NOR | |
| | | month 15<br>↑ H3K9 dimethylation in HIP<br>↓ *BDNF* exon IV mRNA<br>n.c. *BDNF* mRNA in HIP | month 15<br>n.c. BDNF protein in HIP | | month 15<br>↓ neurogenesis in HIP SGZ | month 15<br>n.c. WWM escape latency<br>↓ retention in WWM<br>↓NOR | |
| RMS<br>(pnd 3–12; 3 h daily) | Sprague–Dawley rats | | pnd 56–70<br>n.c. BDNF protein in solitary tract<br>↑ BDNF protein in PVN<br>↓ BDNF protein in phrenic motor nucleus | | pnd 56–70<br>↑ AMPA receptor binding in solitary tract, PVN and phrenic motor nucleus | pnd 56–70<br>↑ hypoxic chemoreflex | Gulemetova et al., 2013 |
| RMS<br>(pnd 3–15; 3 h daily) | Sprague–Dawley rats | | pnd 51<br>↓ BDNF protein in HIP | pnd 51<br>n.c. basal or AS CORT<br>+JS: reduced the effect of JS on decreasing the basal CORT and increasing AS CORT | pnd 51<br>↓ Arc in HIP | | Biggio et al., 2014 |

*(Continued)*

**TABLE 1 | Continued**

| Paradigm | Species | *BDNF* gene regulation | BDNF protein | HPA-axis | Neurogenesis and synaptic plasticity | Behavior | References |
|---|---|---|---|---|---|---|---|
| RMS<br>(pnd 10–15; 3 h daily) | Wistar rats | pnd 16<br>↑ *BDNF* mRNA in cerebral cortex<br>↑ *BDNF* mRNA in cerebellum<br>↓ *BDNF* mRNA in HIP<br>pnd 20<br>↑ *BDNF* mRNA in cerebral cortex<br>n.c. *BDNF* mRNA in cerebellum<br>n.c. *BDNF* mRNA in HIP<br>pnd 30<br>n.c. *BDNF* mRNA in cerebral cortex<br>↑ *BDNF* mRNA in cerebellum (BDNF protein)<br>↑ *BDNF* mRNA in HIP<br>pnd 60<br>↓ *BDNF* mRNA in cerebral cortex<br>n.c. *BDNF* mRNA in cerebellum<br>↑ *BDNF* mRNA in HIP | | | | | Kuma et al., 2004; Lee et al., 2012; Miki et al., 2013, 2014 |
| RMS<br>(pnd 2–21; 3 h daily) | Wistar rats | pnd 60–75<br>↓ *BDNF* mRNA in HIP | | | pnd 60–75<br>↓NCAM and *SYP* mRNA in HIP | pnd 60–75<br>↓ retention in MWM | Aisa et al., 2009 |
| RMS<br>(pnd 2–21; 3 h daily) | Wistar rats | pnd 90<br>↓ *BDNF* mRNA in HIP | | pnd 90<br>↓ GR in HIP<br>↑ CORT | pnd 90<br>n.c. p-Akt, p-GSK3β, p-ERK1, ↓ p-ERK2 in HIP<br>↓ *Arc* mRNA in HIP | pnd 90<br>n.c. OF activity<br>↑ FST immobility<br>↓ retention in MWM<br>↓novel object recognition | Solas et al., 2010 |
| | | month 18<br>↓ *BDNF* mRNA in HIP | | month 18<br>n.c. GR in HIP<br>n.c. CORT | month 18<br>n.c. p-Akt, p-GSK3β, p-ERK1, ↓ p-ERK2 in HIP<br>↓ *Arc* mRNA in HIP | month 18<br>n.c. OF activity<br>↑ FST immobility<br>↓ retention in MWM<br>↓novel object recognition | |
| RMS<br>(pnd 2–22; 3 h daily) | C57Bl/6J mice | pnd 61<br>+ AS: ↓ *BDNF* mRNA | | | | pnd 60–61<br>reduced swim times in FST | MacQueen et al., 2003 |

*↑, increased by the applied maternal separation paradigm; ↓, decreased by the applied maternal separation paradigm; ACTH, adrenocorticotropin; AKT, v-akt murine thymoma viral oncogene homolog; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Arc, activity-regulated cytoskeleton-associated protein; AS, acute stress; BDNF, brain derived neurotrophic factor; CCORT, chronic corticosterone treatment; CORT, corticosterone; Cpu, Caudate Putamen; CS, Chronic stress; DRD2, dopamine receptor D2; DRD3, dopamine receptor D3; ERK, Extracellular-signal-regulated kinases; FST, forced swim test; GR, glucocorticoid receptor; GSK3 β, glycogen synthase kinase 3 beta; HIP, hippocampus; MKP1, mitogen-activated protein kinase phosphatase 1; MWM, Morris Water maze; n.c., not changed by the applied maternal separation paradigm; NCAM, (Neural cell adhesion molecule); NOR, novel object recognition; OF, open field; p-, phosphorylated protein form; PFC, prefrontal cortex; pnd, postnatal day; PVN, Paraventricular nucleus of hypothalamus; RMS, repeated maternal separation; SGZ, subgranular zone; SYP, synaptophysin; VTA, ventral tegmental area.*

regulation of BDNF has been studied extensively in rodent models (**Table 1**).

## Effects of ELS on BDNF mRNA and Protein Expression

There are too few studies to conclude on the direction of changes in BDNF expression levels in the hippocampus or other brain regions (<24 h after first maternal separation onset). One possibility is that BDNF expression decreases acutely after MS. However, pups exposed to MS also experience dietary restriction due to the absence of the mother. It has been demonstrated that dietary restriction increases BDNF expression in the hippocampus, striatum and the PFC in rats (Duan et al., 2001).

In the postweaning period and depending on the characteristics of the MS protocol, increased BDNF expression is reported more consistently. In the period between adulthood and senescence, BDNF expression is reduced, but the time of the switch depends on experimental characteristics (ELS paradigm, stress context in adulthood), sex, rodent strain and brain region of interest.

## Effects of ELS on Epigenetic Regulation of *BDNF*

ELS influences the methylation status of the activity-dependent *BDNF* exon IV expression. One study has shown in rats that repeated maternal separation (RMS) leads to a biphasic effect of the exon IV promoter methylation status. At P21, RMS results in lower H3K9 dimethylation of the exon IV promoter but from adolescence (2 months) into adulthood (15 months), the initially decreased dimethylation after RMS reverses into a significantly increased dimethylation. Low dimethylation status at P21 yields a high *BDNF* exon IV transcription and vice versa during adolescence and adulthood (Suri et al., 2013). In another study where the exon IV promoter methylation change was not confirmed, increased exon IX promoter methylation was reported in hippocampus in maternally separated pups (Kundakovic et al., 2013). In a different ELS paradigm, rat dams with restricted availability of nesting material resulted in reduced maternal licking and grooming behaviors toward their pups and reduced physical interaction between the dams and their offspring (Ivy et al., 2008). This paradigm mimics infant neglect as well, which entails increased basal corticosterone levels in the offspring (Rice et al., 2008). Interestingly, maltreated offspring display hypermethylation of the activity-dependent *BDNF* exon IV promoter region in the PFC, which leads to decreased exon IV expression (Roth et al., 2009). A follow-up study demonstrated additional methylation changes in the hippocampus and amygdala upon exposure to this stress paradigm. These effects were sex and brain region specific (hypermethylation of exon I in males in ventral hippocampus and of exon I in basolateral amygdala (BLA) in females) (Roth et al., 2014). These results were obtained using adult rats and highlight the robust methylation alterations in *BDNF* that occur through ELS. Finally, another study showed that communal nesting of the pups increased histone acetylation at the *BDNF* exon IV promoter (Branchi et al., 2011).

## ELS Effects on Synaptic Plasticity and Behavior

Irrespective of the MS paradigm, neurogenesis in the subventricular zone of the hippocampus appears to be consistently increased in the early postweaning phase and decreased during late adulthood. Moreover, synaptic plasticity related proteins such as neural cell adhesion molecule 1 (NCAM1) and synaptophysin are downregulated during adulthood after MS. Finally, a behavioral phenotype occurs in association with the temporal appearance of the above-mentioned changes, including memory impairment, learned helplessness, reduced social interaction, anhedonia and anxiety.

## Synthesis

These studies indicate that ELS induced alterations of BDNF expression in a brain-region specific and age-dependent manner and provide evidence that BDNF upregulation potentially acts as a neuroprotective mechanism upon ELS exposure.

# INTERPLAY BETWEEN BDNF AND GC

## Effects of BDNF and GC on GR Transcriptome

The GC- and BDNF-signaling pathways influenced by ELS are interlinked throughout life. A recent study confirmed that the GR-specific transcriptome is significantly altered by BDNF. Furthermore, simultaneous treatment of primary rat hippocampal neurons with a synthetic GC, dexamethasone (DEX), and BDNF induces the expression of a unique set of GC-BDNF responsive genes. The majority of these genes are involved in neurite outgrowth and differentiation (Lambert et al., 2013). In the same study, the authors established that BDNF leads to specific phosphorylation of the GR at serines 155 and 287 (Lambert et al., 2013). The latter serine (S287) is stress-hormone responsive, since DEX alone is sufficient to increase phosphorylation. In addition, increased S287 phosphorylation is observed in corticotropin-releasing hormone (CRH) expressing neurons in the parventricular nucleus (PVN) in mice that were exposed to 10 min of forced swim test.

## Impact of BDNF and GCs on Brain Morphology

It is well established that chronic stress affects the morphology of brain structures such as the hippocampus and the amygdala (Watanabe et al., 1992; Magarinos et al., 1996; Vyas et al., 2002). However, questions remain as how stress load and duration affect these brain regions on a structural level. Interestingly, a single exposure to emotional stress has been shown to increase dendritic length and number in amygdala and vice versa in the hippocampus (Rao et al., 2012). However, a recent study suggests that these neuronal phenotypes exclusively occur in rats displaying a vulnerable phenotype, with the degree of cytoarchitectural change predicting the changes in behavioral patterns (Cohen et al., 2014). Based on these findings it is of interest to understand if these stress effects on brain morphology are mediated by GCs. Corticosterone injections over the course

of 3 days led to increased spine formation and concomitant spine elimination. In contrast to these findings, administering daily corticosterone over 10 days caused higher spine elimination (12.1–22.7%), but no increase in spine formation (Liston and Gan, 2011). Interestingly, the developing brain (P30) was even more sensitive. This GC effect seems to be mediated in the CNS directly and preferentially through MR. In another study investigating pubertal rats revealed that a single corticosterone administration evokes differential spatio-temporal effects in the PFC and the BLA (Kim et al., 2014). In particular, 6 days after a moderate dose of corticosterone injection (10 mg/kg) in the medial PFC the dendritic branches and lengths were decreased in parallel with working memory performance. Those effects returned to baseline 1 week after these measurements (day 12). In the BLA the effects of an acute corticosterone injection were slower in onset (day 12 after injection) and were also normalized after a week (day 20). When stress and acute corticosterone administration coincide, they antagonize each other rather than acting in an additive manner (Rao et al., 2012; Cohen et al., 2014).

GC and neurotrophin systems both act in antagonistic as well as in synergistic manners. BDNF and GC are involved in dendritic arborization, whereas BDNF is generally more associated with spine formation and stabilization with GC rather playing an important role in spine turnover (Jeanneteau and Chao, 2013). Mice carrying the minor allele (Met) of the human BDNF Val66Met (rs6265) variant, which alters the structural conformation of the BDNF pro-domain, display less branching in the dentate gyrus (Chen et al., 2006). With regard to GC, one study showed that chronic GC application results in spine loss in the barrel cortex. Interestingly, transient increased GC levels mostly affected newly formed spines, whereas chronically increased GCs affected spines that have been developed early in life (Liston and Gan, 2011).

## Molecular Mechanisms of BDNF and GC Interplay

BDNF can directly influence the HPA-axis regulation through alterations of CRH expression levels. In primary hippocampal neurons, BDNF administration induced a three-fold increase in CRH expression. On the other hand, DEX administration led to repression of CRH, which could not be normalized by BDNF treatment (Jeanneteau et al., 2012). A chromatin immunoprecipitation experiment revealed that DEX treatment evoked increased GR-binding to the CRH promoter (Miller et al., 2011; Jeanneteau et al., 2012). In contrast to DEX, BDNF leads to an increase of cAMP response element-binding protein (CREB) -binding to its site on the CRH promoter, which is in proximity (22 bp) to the GR-binding site. The central mechanistic element in CRH regulation is the recruitment of CREB to the CRH promoter. For transcriptional activity, CREB requires the interaction with a coactivator protein named CREB-regulated transcription coactivator 2 (CRTC2). The increase of GC levels lead to the relocalization of the nuclear CRTC2 to the cytosol and thus decreased CREB transcriptional activity at the CRH promoter (Jeanneteau et al., 2012). In the same study, hypomorphic GR mice had increased BDNF expression and TrkB phosphorylation levels in the PVN in comparison to control littermates. This data is consistent with cross-talk between the neurotrophin and HPA-axis systems through the converging pathways, which are yet to be fully elucidated (**Figure 1**).

Another link between the GC- and BDNF-signaling pathways seems to involve the mitogen-activated protein kinase (MAPK) pathway. Chronic stress not only produces high levels of corticosterone and depressive-like behavior (de Kloet et al., 2005a), but also increases levels of a phosphatase in the MAPK pathway, (i.e., dual specificity phosphatase 1; MKP-1) in the brain. Chronic overexpression of MKP-1 induces detrimental effects by inhibiting axonal growth (Duric et al., 2010). Normalizing GC levels and consecutively MKP-1 expression levels leads to a restoration of stress-related depressive phenotypes through normalization of BDNF expression. Alternatively, constitutive knockdown of MPK-1 is associated with stress-resilience (Jeanneteau et al., 2010).

High GR levels decrease the abundance of the activity-dependent *BDNF* exon IV transcript in the dentate gyrus, CA1 and CA3 regions of the hippocampus without influencing exon I and II transcripts (Smith et al., 1995; Hansson et al., 2006). This calibration effect is corroborated by observations from adrenalectomized mice, in which corticosterone production is abolished and BDNF expression is increased in the CA1, CA3 and dentate gyrus of the hippocampus (Chao et al., 1998). In a further study it was demonstrated that acute GC activity evokes transiently increased tissue-plasminogen activator (tPA) protein levels. The presence of higher levels of tPA yields an increased proteolytic cleavage of pro-BDNF to mature BDNF. The higher amount of mature BDNF itself binds TrkB and enhances downstream MAPK phosphorylation, which is necessary for the formation of enhanced contextual fear memory (Revest et al., 2014).

## CONCLUSION

There is growing body of evidence that the GC–BDNF crosstalk is essential for the early-life programming of the HPA-axis and neurotrophin signaling. During early life, high BDNF and low GC levels are required for neuronal maintenance, synaptic integrity and dendritic spine stabilization in the hippocampus. BDNF-GC equilibrium is crucial throughout life as a major mechanism for stress response regulation. ELS can influence the set point of this equilibrium and thus cause long-term sensitizing effects on stress vulnerability. There is a body of evidence that ELS shifts BDNF as well as GR expression levels in the developing CNS. The long term effect of ELS exposure is a downregulation of BDNF expression (**Table 1**) and GR expression in the hippocampus (Sutanto et al., 1996; Aisa et al., 2007). The combination of both low BDNF and low GR expression favors the vulnerability to develop stress-related disorders during adolescence and adulthood, especially upon additional stress exposures. Phenotypes associated with the ELS-induced reductions of BDNF and GR expression in rodents (**Table 1**) have been additionally observed to be associated with the interaction of the BDNF Met risk allele (Val66Met) and childhood trauma in humans (Molendijk et al., 2012; Aas et al., 2013). Therefore, it is important to understand that genetic and

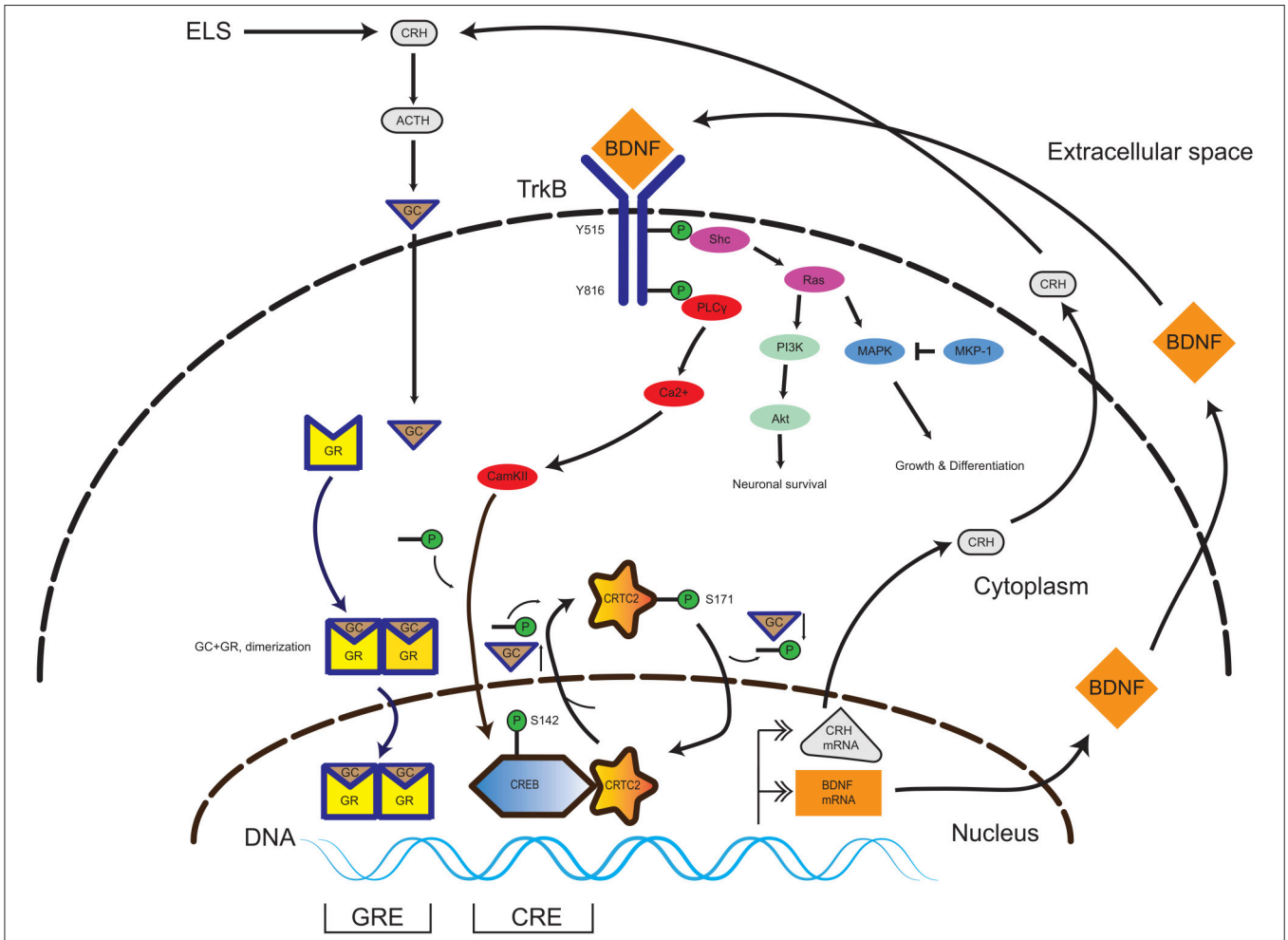


**FIGURE 1 | Interplay of TrkB and GR signaling pathways in the CNS.** In the presence of BDNF, the TrkB receptor homodimerizes and initiates several signaling pathways promoting neuronal survival, growth and differentiation (Akt and MAPK). Activation of the PLCγ pathway leads to CAMKII-mediated phosphorylation of the transcription factor CREB. In presence of a low amount of GC, the CREB-coactivator CRTC2 is dephosphorylated and translocates to the nucleus and binds to phospho-CREB. The phospho-CREB-CRTC2 complex binds at the CRH promoter and drives basal CRH expression in the PVN. Upon occurrence of ELS, the HPA axis signaling pathway is activated, yielding increasing GC levels. GC pass the plasma membrane and enter in to the cytosol and binds to GR, thereby inducing homodimerization (GR-GC complex). The GR-GC complex targets the BDNF promoter and drives basal BDNF production. Exceeding GC levels evoke a translocation of the CREB-coactivator CRTC2 to the cytosol and its phosphorylation, thereby inactivating CREB-dependent CRH production. Thus, the GR and TrkB pathways are calibrated and a specific balance of both GC and BDNF levels is necessary during neurodevelopment to keep homeostasis. Abbreviations: CNS, central nervous system; TrkB, tyrosine kinase receptor type 2; GR, glucocorticoid receptor; CAMKII, Calcium/Calmodulin-Dependent Protein Kinase II; CREB, cAMP Responsive Element Binding Protein; CRTC2, CREB Regulated Transcription Coactivator 2; ELS, early life stress; GC, glucocorticoids; BDNF, brain-derived neurotrophic factor; ACTH, adrenocorticotropic hormone; HPA axis, hypothalamus-pituitary-adrenal gland axis.

epigenetic factors moderate the long term consequences of early adversity (Daskalakis and Binder, 2015). From a therapeutic point of view, preventing steep GC elevations induced by ELS has beneficial effects through constitutive BDNF expression with a concomitant stable, physiological calibration of the GC- and BDNF-signaling pathways.

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# Maternal separation produces alterations of forebrain brain-derived neurotrophic factor expression in differently aged rats

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Early life adversity, such as postnatal maternal separation (MS), play a central role in the development of psychopathologies during individual ontogeny. In this study, we investigated the effects of repeated MS (4 h per day from postnatal day (PND) 1–21) on the brain-derived neurotrophic factor (BDNF) expression in the medial prefrontal cortex (mPFC), the nucleus accumbens (NAc) and the hippocampus of male and female juvenile (PND 21), adolescent (PND 35) and young adult (PND 56) Wistar rats. The results indicated that MS increased BDNF in the CA1 and the dentate gyrus (DG) of adolescent rats as well as in the DG of young adult rats. However, the expression of BDNF in the mPFC in the young adult rats was decreased by MS. Additionally, in the hippocampus, there was decreased BDNF expression with age in both the MS and non separated rats. However, in the mPFC, the BDNF expression was increased with age in the non separated rats; nevertheless, the BDNF expression was significantly decreased in the MS young adult rats. In the NAc, the BDNF expression was increased with age in the male non-maternal separation (NMS) rats, and the young adult female MS rats had less BDNF expression than the adolescent female MS rats. The present study shows unique age-differently changes on a molecular level induced by MS and advances the use of MS as a valid animal model to detect the underlying neurobiological mechanisms of mental disorders.

**Keywords: maternal separation, brain-derived neurotrophic factor (BDNF), medial prefrontal cortex (mPFC), hippocampus**

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## Introduction

Adverse early life events are considered to be risk factors for the development of psychiatric diseases (Walker and Diforio, 1997; Ellenbroek and Cools, 1998; Marais et al., 2008; Réus et al., 2011). In rats, maternal separation (MS), which deprives pups of their mothers, has often been used as a model for early life adversity (Hall, 1998; Marco et al., 2009). MS has been demonstrated to induce behavioral and cognitive abnormalities, such as increased depressive and anxiety-like behaviors (Marais et al., 2008; Jia et al., 2009; Rentesi et al., 2010) and prepulse inhibition (PPI) deficits (Ellenbroek and Cools, 1998, 2002). MS has also been shown to decrease new born cells in the hippocampus and the granular cell number in the dentate gyrus (DG) of juvenile and adult rats (Mirescu et al., 2004; Oreland et al., 2010; Hulshof et al., 2011); these findings suggest that MS can affect the neuroplasticity of rats.

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family (Hyman et al., 1991) and exerts a wide range of functions, such as maintaining neuronal survival, structure, growth, and differentiation and promoting synaptic plasticity of learning and memory (Fumagalli et al., 2007; Pillai and Mahadik, 2008). BDNF has also been implicated in the neurobiological mechanisms of psychiatric diseases (Weickert et al., 2003; Schneider et al., 2011; Favalli et al., 2012). BDNF expression in multiple brain regions is sensitive to adverse life experiences. For example, our lab has reported that adolescent social isolation affects BDNF levels in the medial prefrontal cortex (mPFC), Nucleus accumbens (NAc) and hippocampus of adult rats (Han et al., 2011; Meng et al., 2011). Several studies have reported the effects of MS on BDNF levels in different brain areas; however, the results have been inconsistent. For example, MS increased the BDNF level in the hippocampus of adult rats (Greisen et al., 2005; Faure et al., 2007), reduced the BDNF levels in the PFC, hippocampus and striatum of mice (Ognibene et al., 2008) or had no change with respect to the BDNF levels in the PFC and hippocampus (Réus et al., 2011). These discrepancies may be resulted from the different experimental procedures, species and strains adopted in these studies.

Furthermore, other studies have indicated that the developmental period of animals may be another important factor for MS effects. For example, Roceri et al. (2004) reported that MS produced a short-term-up-regulation of the BDNF level in the hippocampus and PFC on postnatal day (PND) 17 and a reduction of BDNF expression in the PFC in adulthood. Kuma et al. (2004) conformed that MS decreased the BDNF mRNA expression on PND 16 and increased the BDNF mRNA expression on PND 30 and 60 in the hippocampus of rats, and there was no significant difference between MS and non-maternal separation (NMS) rats on PND 20. Although these studies mentioned above suggested the developmental factors of MS effects, to date, there is not any studies that investigated the effects of MS on forebrain BDNF expression in juvenile, adolescent and young adult rats systematically.

In the present study, we aimed to investigate the effects of repeated MS (4 h/day from PND 1–21) on the BDNF expression levels in the mPFC, NAc and hippocampus in juvenile (PND 21), adolescent (PND 35) and young adult (PND 56) rats. The three brain regions chosen in this study were based on the close functional relationships with BDNF activity of them.

## Material and Methods

### Animals

Male and female Wistar rats were obtained from the Academy of Chinese Military Medical Science. All of the animals were housed on a 12 h light/12 h dark cycle (lights on at 7:00 a.m.) and with free access to food and water. The environmental conditions was kept constant (ambient temperature 22°C). All experimental procedures were performed in strict accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) and approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University.

### Maternal Separation

The protocol of MS were adapted from previous studies (Li et al., 2013; Wang et al., 2015). The male and female rats were mated to produce litters that consisted of 8–12 pups. After birth, the pups were randomly divided into two groups: the MS (MS group, 48 pups) and the NMS (NMS group, 48 pups). MS was performed on MS group while the NMS group was undisturbed from PND 1–21. Each group had 24 male and 24 female pups. During the separation, the pups in MS group were separated from their mothers for 4 h (10:00–14:00) per day from PND 1–21 and maintained on heated sawdust (29 $\pm$ 1°) separately from their littermates. The dams of the pups in MS group were left in the home cage during the separation. The pups in NMS group remained in their home cage with their mothers and littermates during the 4 h separation. After weaning at PND 21, 16 MS (8 males and 8 females) and 16 NMS (8 males and 8 females) animals were sacrificed for the BDNF measurement by immunohistochemistry (IHC). The rest of rats were reallocated to different cages (4 rats per cage). Then, at PND 35, another 16 MS (8 males and 8 females) and 16 NMS (8 males and 8 females) animals were sacrificed for the BDNF measurement by IHC. At PND 56, the last 16 MS (8 males and 8 females) and 16 NMS (8 males and 8 females) animals were sacrificed for the BDNF measurement by IHC.

### Immunohistochemistry

This procedure IHC has been described in previous studies (D'andrea et al., 2001; Xavier et al., 2005; Han et al., 2011; Meng et al., 2011). Briefly, the rats were anesthetized of intraperitoneal (ip) administration with chloral hydrate (400 mg/kg) and perfused with phosphate buffered saline (PBS, 0.01 M) followed by 4% paraformaldehyde dissolved in PBS. The brain regions of interest (mPFC: 5.70–2.70 mm from bregma; NAc: 2.70–0.70 mm from bregma; hippocampus: −1.30 to −5.30 mm from bregma; Paxinos and Watson, 2006) were dissected on ice using a rat brain mold and post-fixed by 4% paraformaldehyde for 6 h. Then the brain samples were dehydrated (3 × 30 min 70%, 90%, 96%, 100% ethanol and Roti-Histol) and embedded in paraffin. Next, the paraffin which containing the brain samples were cut into sections (4 μm) using a microtome (Leica 235), then the sections were pasted onto slides and dried (30 min, 58°C) on a heating plate. After washing in 0.05 M PBS (3 × 2 min), the sections were put into citrate buffer solution and heated in microwave oven. Afterwards, the slides were blocked (10% goat serum and 1% BSA dissolved in 0.01 M PBS, 20 min at room temperature, RT) and incubated in a first rabbit anti-BDNF polyclonal IgG (1:200, Santa Cruz Biotechnology, overnight at 4°C). After washing in 0.05 M PBS (3 × 5 min, RT), the sections were incubated with a secondary goat anti-rabbit IgG (1:1000, Santa Cruz Biotechnology, 1 h at RT), and incubated in an avidin–biotin–peroxidase complex (1 h). Finally, the sections were dehydrated by serial alcohol rinsing, dewaxed in dimethyl benzene, and cover-slipped.

### Quantification and Statistical Analyses

The slides were viewed and photographed using a light microscope (Olympus BX-51), and the images were analyzed using a software (Image-pro plus 6.0). The BDNF levels were estimated by counting all of the BDNF-positive cells present in two serial sections interspaced by 4 μm in the middle of the mPFC, NAc and hippocampus. The areas of the mPFC, NAc and the CA1, CA2/3 and DG of the hippocampus were measured, and the number of units per 1 $mm^2$ was calculated bilaterally per rat. For the analysis, the cell counts were averaged into a single score for each rat.

All of the data are shown as the mean ± standard error of the mean (SEM). The analyses were performed using the SPSS 16 software. The IHC results were analyzed using a multivariate analysis of variance (MANOVA). The comparisons with two and three groups were analyzed using Student's *t*-test and a one-way ANOVA followed by least significant difference (LSD) *post hoc* tests, respectively. The significance level was defined as $p < 0.05$.

## Results

### Effects of MS on the BDNF Expression in the Hippocampus

The results of the BDNF expression in the CA1 were summarized in **Figure 1**. The results showed that there were significant main effects of MS ($F_{(1,84)} = 7.987$, $p = 0.006$) and age ($F_{(2,84)} = 7.421$, $p = 0.001$), but not gender. The interaction between MS and age was significant ($F_{(2,84)} = 5.385$, $p = 0.006$), whereas the other interactions were not significant. Further analysis (*t*-test) indicated that MS increased the BDNF expression in the CA1 in the PND 35 rats ($t_{(30)} = 4.035$, $p < 0.001$; **Figure 1A**). A one-way ANOVA revealed that there was a significant difference among the three ages in both the NMS and MS groups (NMS: $F_{(2,45)} = 8.211$, $p = 0.001$; MS: $F_{(2,45)} = 5.099$, $p = 0.010$). The *post hoc* (LSD) comparisons revealed that in the NMS group, the PND 35 and PND 56 rats had significantly less expression compared with the PND 21 rats; in the MS group, the PND 56 rats had significantly less expression compared with the PND 21 and PND 35 rats (**Figure 1A**).

In the CA2/3, the overall effects of MS, age and gender were not significant; all of the interactions were also not significant.

The BDNF expression in the DG was summarized in **Figure 1B**. MS resulted in an overall increased BDNF protein expression in the DG ($F_{(1,84)} = 7.741$, $p = 0.007$). The overall effects of age and gender were not significant, and all of the interactions were also not significant. Further analysis (*t*-test) showed that MS significantly increased the BDNF expression in the DG in the PND 35 and PND 56 rats (PND 35: $t_{30} = 2.350$, $p = 0.026$; PND 56: $t_{30} = 2.169$, $p = 0.038$).

### Effects of MS on the BDNF Expression in the mPFC

The BDNF expression in the mPFC for each group was summarized in **Figure 2**. MS significantly decreased the BDNF expression in the mPFC ($F_{(1,84)} = 4.006$, $p = 0.005$). The influence of age and gender on the expression of BDNF was not significant. The interaction between MS and age was significant ($F_{(2,84)} = 7.749$, $p = 0.001$), whereas the other interactions were not significant. Further analysis (*t*-test) indicated that MS reduced the BDNF expression in the mPFC in the PND 56 rats ($t_{(30)} = 5.350$, $p < 0.001$; **Figure 2A**). A one-way ANOVA revealed that there was a significant difference among the three ages in both the NMS and MS groups (NMS: $F_{(2,45)} = 3.238$, $p = 0.049$; MS: $F_{(2,45)} = 4.607$, $p = 0.015$). The *post hoc* (LSD) comparisons revealed that in the NMS group the PND 56 rats had significantly increased expression compared with the PND 35 rats; in the MS group the PND 56 rats had significantly less expression compared with the PND 21 and PND 35 rats (**Figure 2A**).

### Effect of MS on the BDNF Expression in the NAc

In the NAc, the overall effects of MS, age and gender were not significant; however, there was a significant interaction among MS, age and gender ($F_{(2,84)} = 4.475$, $p = 0.014$). The other interactions were not significant. Further analysis (one-way ANOVA) revealed that for the male rats there was a significant difference among the three ages in the NMS, but not in the MS groups (NMS: $F_{(1,22)} = 4.378$, $p = 0.026$; MS: $F_{(1,22)} = 1.613$, $p = 0.223$). However, for the female rats there was a significant difference among the three ages in the MS, although not in the NMS groups (NMS: $F_{(1,22)} = 0.970$, $p = 0.395$; MS: $F_{(1,22)} = 4.564$, $p = 0.023$). The *post hoc* (LSD) comparisons revealed that in the male NMS group the PND 21 rats had significantly less expression compared with the PND 35 and PND 56 rats (**Figure 3A**); in the female MS group the PND 56 rats had significantly decreased BDNF expression compared with the PND 35 rats (**Figure 3B**).

## Discussion

The current findings showed that repeated MS has fundamental effects on BDNF protein expression in the forebrain of juvenile, adolescent and young adult male and female rats. MS increased BDNF expression in hippocampus of rats but decreased it in the mPFC. BDNF expression in the CA1 was decreased with age. However, in the mPFC, the increased expression of BDNF with age in non-separated rats was reversed in MS rats. The effect of gender on BDNF expression was only found in the NAc.

Firstly, our results suggested that there were different effects of MS on BDNF expression in the mPFC, hippocampus and NAc. MS increased in the hippocampus of adolescent and young adult rats and decreased in the mPFC of young adult rats. Similarly, one previous study reported that MS produced a short-term-up-regulation of BDNF expression in hippocampus and PFC, which was measured on PND 17, and a reduction of BDNF expression in the PFC in adulthood (Roceri et al., 2004). Our results indicated that MS did not affect the expression of BDNF in NAc. The alike results were also found in our previous study in adult rats (Xue et al., 2013). These findings reminded us that MS may affect BDNF expression differently in the mPFC, hippocampus and NAc.

Since BDNF was closely related to both neural plasticity and cytoarchitecture, are the influences of MS has on neural

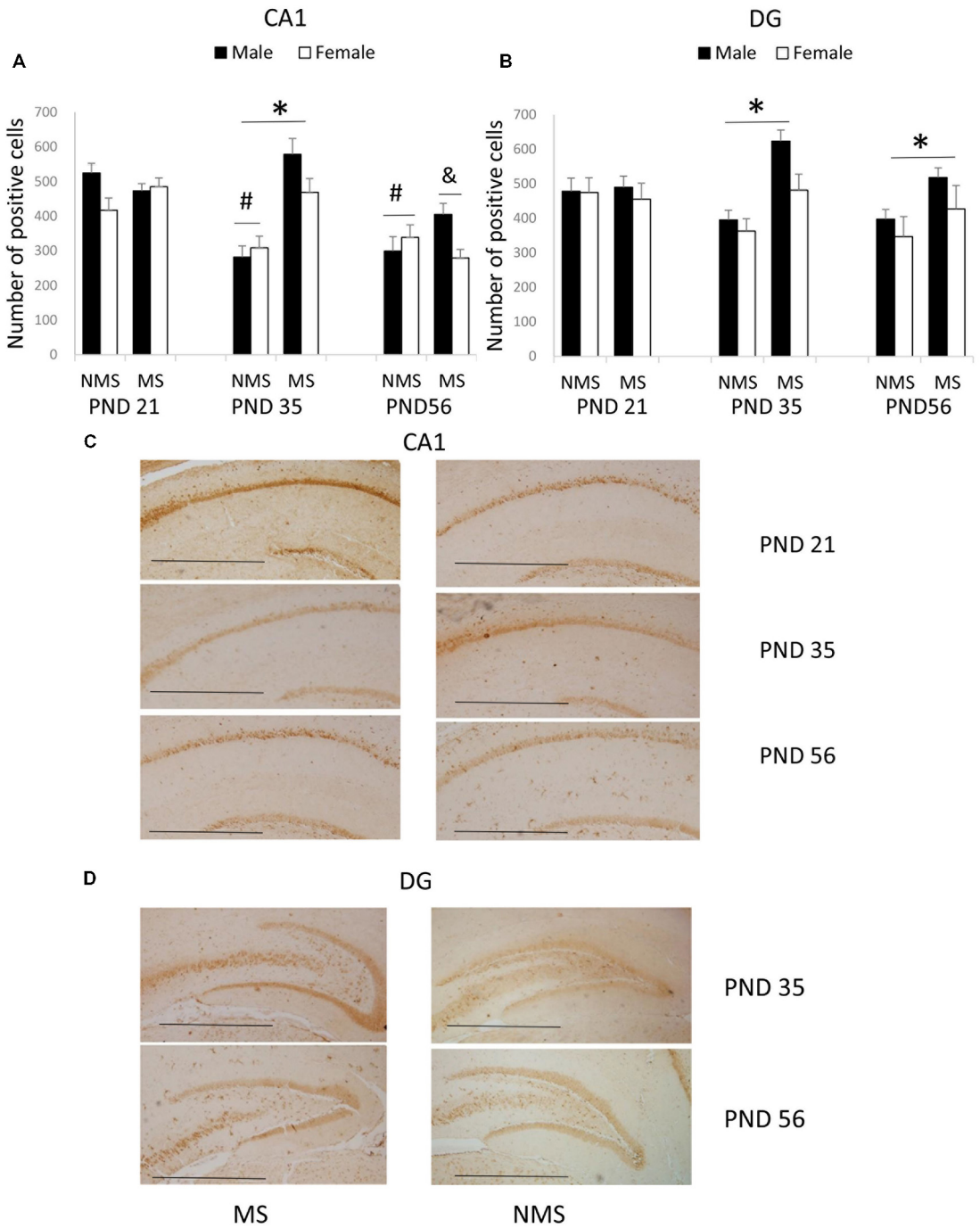


**FIGURE 1 | Effects of Maternal separation (MS) on the brain-derived neurotrophic factor (BDNF) expression in the hippocampus, including the effects of MS on the BDNF expression at different ages in CA1 (A) and dentate gyrus (DG) (B) and representative immunohistochemistry (IHC) figures of CA1 (C) and DG (D).** The results are expressed as the mean ± S.E.M. (*compared with PND 35 NMS rats, $p < 0.05$; #compared with PND 21 NMS rats, $p < 0.05$; &compared with PND 21 and PND 35 MS rats, $p < 0.05$). Scale bar = 250 μm.

plasticity and cytoarchitecture in mPFC and hippocampus similar? Previous study reported that MS (3 h per day from PND 2–14) increased the hippocampal neurogenesis which was assessed by using BrdU and DCX, meanwhile, the histone methylation at the *BDNF* IV promoter and the expression of BDNF were also increased in hippocampus (Suri et al., 2013). MS also increased CREB and BDNF levels and hippocampus progenitor proliferation in hippocampus (Nair et al., 2007). Another study demonstrated that MS (2 h per day from PND 1–12) decreased the dendritic length and dendritic spine density

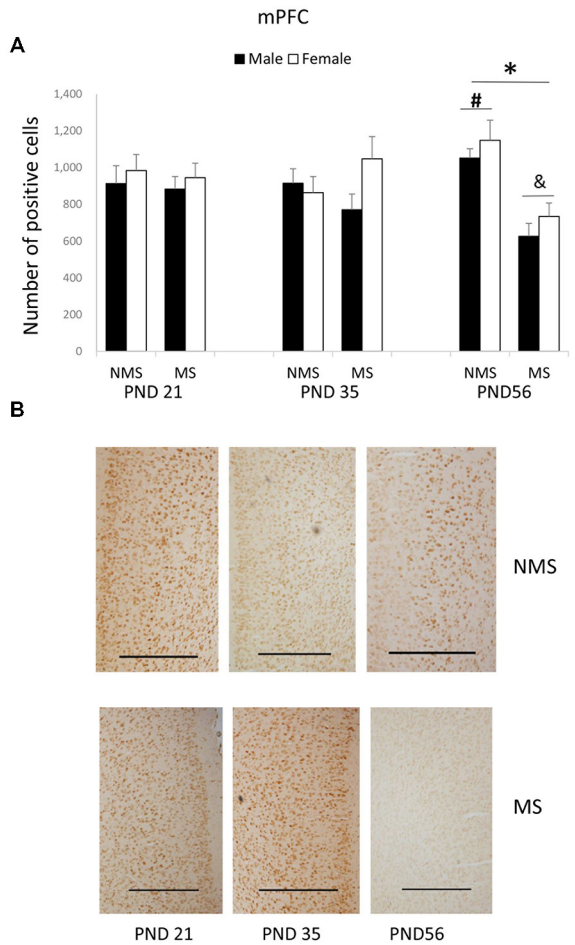


**FIGURE 2 | Effects of MS on the BDNF expression in the mPFC, including the effects of MS on the BDNF expression at different ages (A) and representative IHC figures (B).** The results are expressed as the mean ± S.E.M. (*compared with PND 56 NMS rats, $p < 0.05$; #compared with PND 35 NMS rats, $p < 0.05$; &compared with PND 21 and PND 35 MS rats, $p < 0.05$). Scale bar = 500 μm.

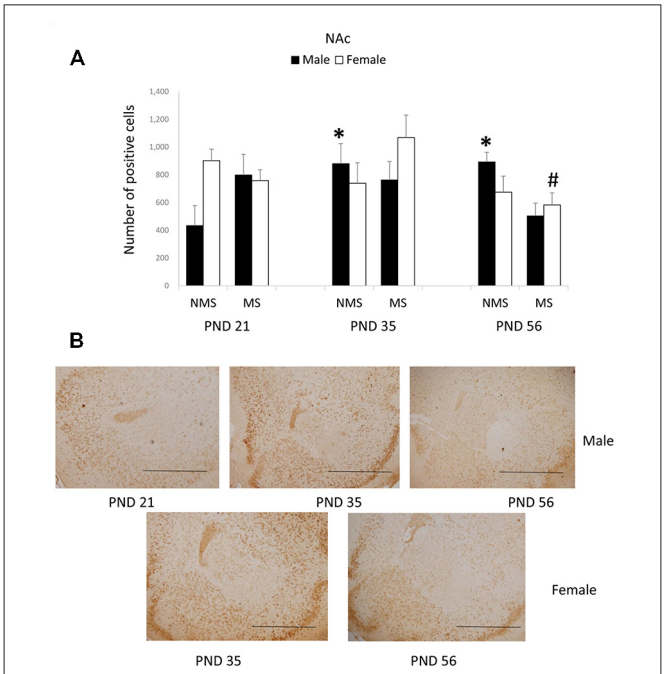


**FIGURE 3 | Effects of MS on the BDNF expression in the Nucleus accumbens (NAc), including the effects of age on the BDNF expression in different gender rats (A) and the representative IHC figures (B).** The results are expressed as the mean ± S.E.M. (*compared with PND 21 male NMS rats, $p < 0.05$; #compared with PND 35 female MS rats, $p < 0.05$). Scale bar = 500 μm.

of the neuron in PFC (Monroy et al., 2010). MS (24 h on PND 9) also induced the reduction in the thickness of PFC and of the density of NeuN-immunolabeled neurons in PFC of rats (Aksić et al., 2013). These studies on cytoarchitecture and neural plasticity supported our findings about changes on BDNF levels in PFC and hippocampus induced by MS.

In addition, existing studies have shown that the inconsistency of MS effects was also reflected in cognitive function, such as the spatial learning and reversal learning in Morris water maze (MWM). Our recent study reported that repeated MS (4 h per day from PND 1–21) increased swim distance in spatial learning and decreased escape latency in reversal learning of MWM in adolescent and early adult rats (Wang et al., 2015). MS could induce the impairment of spatial learning of MWM in adolescent (Frisone et al., 2002) and adult rats (Garner et al., 2007), and also could enhance the performance in reversal learning of MWM in adult rats (Lehmann et al., 1999). Hippocampus, which was a key brain region of memory, played an important role in cognitive function in MWM. Specifically deleted BDNF in hippocampus impaired the spatial learning in MWM (Heldt et al., 2007). PFC was also closely associated with spatial working memory in MWM (Xing et al., 2012). These behavioral and BDNF results suggested that differences among cognitive abnormalities of MS animals may be related to the diverse changes in hippocampus and mPFC, but more researches are still needed.

Secondly, as we have noted above, the developmental period of animals may be another important factor for the MS effects. The present study investigated the effects of MS on BDNF expression in the hippocampus and mPFC of juvenile, adolescent and young adult rats. Our results found that MS did not change the BDNF expression in the CA1 and DG of PND 21 rats, but increased the BDNF expression in the CA1 and DG of PND 35 rats, as well as in the DG of PND 56 rats. Similarly, another previous study indicated that MS (3 h per day from PND 10–15) decreased the expression of BDNF mRNA in the hippocampus of PND 16 rats and increased the expression of BDNF mRNA in the hippocampus of PND 30 and 60 rats. What's more, it was reported that there was no significant difference between MS and mother-reared control rats on PND 20 (Kuma et al., 2004). These findings were consistent with the results in the present study.

Thirdly, regarding the effects of MS on male and female rats, the present study found that the expression of BDNF in the mPFC and hippocampus was not significantly different between the male and female rats. These results reminded us that the

expression of BDNF, which was affected by MS, was not different between the male and female rats. Marco et al. (2013) also found that MS decreased the expression of BDNF in the PFC and hippocampus both in male and female adolescent rats, but MS increased the expression of glial fibrillary acidic protein (GFAP) in male adolescent rats, not in female rats. However, our results showed that between the MS and NMS rats of different ages the effect of gender on BDNF expression in the NAc was different. In the male NMS rats BDNF expression was increased with age. A recent study reported that male juvenile rats (PND 26–28) exhibited significantly elevated basal BDNF expression in the NAc compared with their male adult (4 months) counterparts (Perreault et al., 2013). These results were different with our findings and this discrepancy may be because we did not observe data at 4 months after birth. In the female MS group the BDNF expression of the young rats was decreased compared with the adolescent rats. No comparable result was reported in the previous study.

Fourthly, the present study also found that the expression of BDNF in the CA1 was decreased with age in the NMS rats, and MS did not affect a change with age. A previous study reported that the expression of BDNF in the hippocampus changed with age: the expression of BDNF increased after birth and it reached the highest level in the second week after birth. After that, the expression of BDNF gradually declined with age (Silhol et al., 2005). These findings, consistent with our results, revealed the effect of age on the process of BDNF neural-development in the brain.

More importantly, the present study found that compared with juvenile and adolescent rats, the BDNF expression in the mPFC of young adult rats was significantly affected by repeated MS. The highest BDNF expression in the NMS young adult rats was reversed to the lowest expression of the MS rats. These results suggested that the increase of BDNF expression in the mPFC for young adult rats may be stopped and reversed by MS, and no comparable result was reported in previous studies. However, our lab had reported that repeated MS (4 h per day from PND 1–21) improved reversal learning of the MWM in young adult rats. For NMS rats, compared with juvenile or adolescent rats, young adult rats had significantly decreased cognitive flexibility; for MS rats, MS significantly improved reversal learning of young adult rats (Wang et al., 2015). Most previous studies had reported a positive relationship between BDNF expression in the PFC and cognitive function (Bredy et al., 2007; Sakata et al., 2013). However, a recent report found that stress facilitated reversal learning of mouse, and ventromedial prefrontal cortex (vmPFC) lesions mimicked this effect of stress, but this enhanced reversal learning of mouse induced by stress was prevented by BDNF infusion into the vmPFC (Graybeal et al., 2011), which in part supported our findings. Altogether, the relationship between cognitive function and BDNF expression in the mPFC requires further investigation.

Epigenetics research also confirmed the influence of early life adversity on BDNF expression in brain. Roth et al. (2009) demonstrated that early maltreatment increased the methylation of *BDNF* DNA and it reduced the BDNF expression in PFC of adult rats. Furthermore, many studies have confirmed that MS led to hyperactivity of hypothalamic-pituitary-adrenal (HPA) axis (Plotsky and Meaney, 1993; Lehmann et al., 2002; Lippmann et al., 2007). MS reduced the level of glucocorticoid type-2 and corticotropin-releasing hormone type-1 receptor (CRH1) mRNA in hippocampus. MS also impaired the memory function and decreased the expression of glucocorticoid in hippocampus of rats (Llorente et al., 2011). These biochemical changes may contribute to the neurobiological foundation of behavioral and cognitive alterations induced by MS, which should be further detected in our future research.

## Conclusion

These present findings suggest that repeated MS induced different types of forebrain neurobiological changes in juvenile, adolescent, and young adult rats, revealing the varying patterns of BDNF expression along with age in different brain regions and indicating that the influence of gender was only embodied in NAc not mPFC or hippocampus. The present study provided new evidence for the study of behavioral and neuro-biochemical alterations induced by adverse early life event. Considering the close relationship between human BDNF and early life adversity, these findings have potential clinical implication for treating mental disorders.

## Author Contributions

FS designed the research; QW performed the research and acquired the data; QW, FS and WW interpreted and analyzed the data; and QW, FS and WW drafted, revised and wrote the paper.

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# Early-life stress induces persistent alterations in 5-HT$_{1A}$ receptor and serotonin transporter mRNA expression in the adult rat brain

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Early-life experience plays a major role in the stress response throughout life. Neonatal maternal separation (MS) is an animal model of depression with an altered serotonergic response. We hypothesize that this alteration may be caused by differences in 5-HT$_{1A}$ receptor and serotonin transporter (SERT) mRNA expression in brain areas involved in the control of emotions, memory, and fear as well as in regions controlling the central serotonergic tone. To test this, Sprague–Dawley rats were subjected to MS for 3 h daily during postnatal days 2–12. As control, age matched rats were non-separated (NS) from their dams. When animals reached adulthood (11–13 weeks) brain was extracted and mRNA expression of 5-HT$_{1A}$ receptor in amygdala, hippocampus and dorsal raphé nucleus (DRN) and SERT in the DRN was analyzed through *in situ* hybridisation. Densitometric analysis revealed that MS increased 5-HT$_{1A}$ receptor mRNA expression in the amygdala, and reduced its expression in the DRN, but no changes were observed in the hippocampus in comparison to NS controls. Also, MS reduced SERT mRNA expression in the DRN when compared to NS rats. These results suggest that early-life stress induces persistent changes in 5-HT$_{1A}$ receptor and SERT mRNA expression in key brain regions involved in the development of stress-related psychiatric disorders. The reduction in SERT mRNA indicates an alteration that is in line with clinical findings such as polymorphic variants in individuals with higher risk of depression. These data may help to understand how early-life stress contributes to the development of mood disorders in adulthood.

**Keywords: maternal separation, 5-HT$_{1A}$ receptor, serotonin transporter, amygdala, dorsal raphé nucleus**

## INTRODUCTION

In the early postnatal period of the rat, the brain is thought to be a developmental equivalent to the last trimester *in utero* and the perinatal period of human brain development (Romijn et al., 1991; Watson et al., 2006; Goodfellow et al., 2009), thus allowing for the use of postnatal rodent models in the investigation of the early programing of stress-related psychiatric disorders. It has been proposed that stress during developmental stages, can lead to developmental alterations that become evident in adult life (Barker, 1995), and moreover, during early-life the psychosocial milieu can substantially alter the nervous system, through mechanisms that permanently affect gene expression (Mathews and Janusek, 2011).

In the central nervous system (CNS), one of the key neurotransmitter systems involved in the response to stress and in the development of neuropsychiatric disorders is the 5-hydroxytriptamine (5-HT) system (Kirby et al., 1995; Graeff et al., 1996; Cryan et al., 2005; Savitz et al., 2009; O'Leary and Cryan, 2010). The majority of 5-HT neurons are located in the dorsal and median raphé nucleus in the brainstem (Graeff et al., 1996; Michelsen et al., 2007). Projections from these neurons innervate several structures of the limbic system, including the amygdala and hippocampus, and it has been described that through these projections the 5-HT system regulates the fight or flight reaction to stress (Graeff et al., 1996; Michelsen et al., 2007), by a region-specific release of 5-HT (Kreiss and Lucki, 1994; Kirby et al., 1995; Graeff et al., 1996).

There are 14 types of 5-HT receptors, divided into seven families, with different subtypes identified by letters (A–F in the case of 5-HT$_1$ receptors; Hoyer et al., 1994; Barnes and Sharp, 1999; Bockaert et al., 2010). One of these receptors is 5-HT$_{1A}$, a G protein-coupled receptor that has been described to play an important role in the development of psychiatric disorders (Bowen et al., 1989; López et al., 1998; Drevets et al., 1999; Gross et al., 2002; Savitz et al., 2009). The 5-HT$_{1A}$ receptor is predominantly a somatodendritic autoreceptor in the neurons of the raphé nucleus regulating the amount of 5-HT released and therefore serotonergic activity in the different projection areas (Blier and de Montigny, 1987; Hutson et al., 1989; Hjorth and Sharp, 1991; Kreiss and Lucki, 1994; Savitz et al., 2009). Also, 5-HT$_{1A}$ receptor expression has been described in forebrain areas (Chalmers and Watson, 1991; Pompeiano et al., 1992; Cryan et al., 2005; Savitz et al., 2009) including the hippocampus and amygdala, structures involved in learning, control of emotions, memory and fear related information (Vizi and Kiss, 1998; Nestler et al., 2002; LeDoux, 2007).

Alterations in 5-$HT_{1A}$ receptor function have been related to mood disorders, as imaging analysis shows that depressive patients have reduced 5-$HT_{1A}$ receptor binding (Drevets et al., 1999; Sargent et al., 2000) as well as blunted responses to 5-$HT_{1A}$ receptor agonists (Lesch et al., 1990a,b).

Another component of the 5-HT system is the serotonin transporter (SERT), a presynaptic protein involved in the termination of the serotonergic signal through the reuptake of 5-HT from the synapse (Blakely et al., 2005). In the pharmacological treatment of depression, selective serotonin reuptake inhibitors (SSRIs) have been widely used (Frazer, 1997). SSRIs can readily inhibit SERT activity and elevate the serotonergic tone in the brain. However, full therapeutic effects become apparent only after chronic SSRI use, suggesting that alterations in this transporter are highly relevant to the development and treatment of psychiatric disorders (Frazer and Benmansour, 2002).

Neonatal maternal separation (MS) is a well validated animal model of depression and increases anxiety resulting in behavioral alterations (Lippmann et al., 2007) and functional changes in the hypothalamus-pituitary-adrenal (HPA) axis responsiveness in adulthood (Ladd et al., 1996; Schmidt et al., 2004; O'Mahony et al., 2009). In addition, MS animals have been reported to display alterations in their central corticotrophin releasing factor (CRF) system (Bravo et al., 2010; O'Malley et al., 2011), which is suggestive of an altered gene expression in key brain areas as result of early-life stress.

There is evidence suggesting an enhanced serotonergic response in animals subjected to MS, as there are differences in brain stem levels of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA; O'Mahony et al., 2008), as well as increased responsiveness to the SSRI citalopram (Arborelius et al., 2004).Therefore, differences in central serotonergic modulation in adult rats subjected to early-life stress could arise as a result of altered 5-$HT_{1A}$ receptor and SERT expression in areas of the brain involved in the control of emotions, memory, and fear as well as in areas controlling the central serotonergic tone. To test this, *in situ* hybridization was used to study topographical differences in 5-$HT_{1A}$ receptor and SERT mRNA expression in the hippocampus, amygdala, and dorsal raphé nucleus (DRN) between MS and non-separated (NS) rats.

## MATERIALS AND METHODS

### ANIMALS

Adult male Sprague–Dawley (SD) rats that underwent a MS protocol were used ($n = 6$ MS from three different litters and $n = 6$ NS controls from three different litters). All animals were housed in standard conditions (room temperature of 21°C, with a 12 h light dark cycle) with access to regular chow and water *ad libitum*. Cages were cleaned once weekly to avoid excessive handling. Rats were of comparable weight (276–410 *g*) and age (11–13 weeks) at the moment of sacrifice All experimental procedures were carried out in accordance with the protocols approved by the Ethics Committee, at University College Cork, Cork, Ireland under a license issued from the Department of Health and Children (Cruelty to Animal Act 1876, Directive for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [89/609/EEC]).

### MATERNAL SEPARATION

Early-life stress procedure (Hyland et al., 2009; O'Mahony et al., 2009, 2010) was adapted from a previously described protocol (Wigger and Neumann, 1999). Briefly, the litters that were randomly assigned to undergo MS, were removed from the home cage and placed into a smaller cage on heating pads set at 30–33°C for 3 h (9.00–12.00 h). After that time, pups were returned to the original home cage in the main colony room. This procedure was repeated from postnatal day 2 (P2) to P12. Control, NS litters remained undisturbed except for routine cage cleaning performed once a week. At P21, pups were weaned and group-housed (3–5 per cage), and left undisturbed until adulthood (11–13 weeks). We have previously shown that this MS protocol induces an array of behavioral and physiological changes that are indicative of increased anxiety and altered HPA axis function (O'Mahony et al., 2009).

### SACRIFICE AND *IN SITU* HYBRIDISATION

Animals were lightly anesthetized with isoflurane, and killed by decapitation. The brain was immediately extracted and snap frozen in isopentane kept cold with dry ice. The brains were stored at −80°C before being processed for *in situ* hybridisation.

The *in situ* hybridisation was carried out with oligodeoxynucleotide (cDNA) probes complementary to 5-$HT_{1A}$ receptor mRNA (2107–2151 pb access number AF217200)and SERT mRNA (1719–1763 pb access number Y11024.1), labeled with a digoxigenin (DIG) oligonucleotide 3′-OH tailing kit (Roche, Molecular Biochemicals, Mannheim, Germany). The hybridisation was conducted as previously described (Bravo et al., 2009, 2010). Briefly, coronal brain sections of 10 μm thick were obtained from frozen brains and mounted on superfrost-plus glass slides (Menzel-Glaser, Menxel GmbH & Co., Germany). For hippocampus, four to five non-consecutive slices separated at least 100 μm from each other, approximately from bregma −2.56 mm to bregma −3.6 mm were analyzed bilaterally. For the amygdala: bilateral analysis of four to five slices of tissue, separated at least 100 μm from each other, approximately from bregma −1.80 mm to bregma −2.80 mm. In the case of the DRN at least three slices separated as a minimum as 100 μm from each other, approximately from bregma −7.64 mm to bregma −8.00 mm were obtained. These sections were post-fixed in 4% paraformaldehyde made in PBS for 30 min. Then the slides were permeabilized with proteinase K (0.5 mg/100 mL in TE buffer) and treated with acetic anhydride buffer. Next, the slides underwent dehydration through a series of ethanol dilutions (70, 95, and 100%) before being delipidated in chloroform for 5 min. The tissues were then rehydrated and placed in a humidity chamber with the hybridisation solution [formamide 50%, saline sodium citrate (SSC) buffer 4x, sheared salmon DNA 6.25 mg/mL, tRNA 125 μg/mL, and cDNA probe at fixed concentration of 100 pmol/mL for each probe] and incubated overnight at 37°C. After that, the sections were washed in ascending dilutions of SSC buffer (4, 2, 1, and 0.5x), and then equilibrated with maleic acid 0.1 M buffer before blocking for unspecific protein binding with Roche's blocking reagent (Roche, Molecular Biochemicals, Mannheim, Germany). After 30 min of blocking, the DIG molecules attached to the hybridized probes were detected with an anti DIG antibody,

conjugated with an alkaline phosphatase (Roche, Molecular Biochemicals, Mannheim, Germany). Finally, a substrate for the alkaline phosphatase (NBT/BCIP; Sigma, St. Louis, MO, USA) was added, and when a violet/blue precipitate was present on the tissues, the reaction was stopped. The slides were then left to air dry and cover-slips were mounted with DPX mounting media (Fisher Scientific, Loughborough, UK). Once the mounting media was dry, pictures of the areas of interest were taken with an Olympus DP71 digital camera attached to an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan). Specificity of the hybridisation was evaluated by the use of 100-fold excess of the unlabelled oligodeoxynucleotide. For semiquantitative analysis, densitometric measurements of each hippocampal, amygdala, and DRN were analyzed using FujiFilm's Science Lab Multi Gauge v2.2 software (Fuji Photo Film Co., Ltd). All pictures were analyzed in gray scale and the value given by the software corresponds to the intensity of pixels (the darkest staining is the highest intensity; and the lightest staining the lowest intensity) in a given area (density of pixels). In the hippocampus, the hybridisation signal in the *stratum radiatum* was considered as background and was subtracted from the pixel density values obtained in the hippocampal cell layers. As for the amygdala, a small region between the analyzed areas was considered as background, and for the DRN a small region surrounding this structure was taken as background. For each animal the value represents the average from 4–5 non-consecutive brain sections (analyzed on both brain hemispheres for hippocampus and amygdala).

### STATISTICAL ANALYSIS

All the values are expressed as the mean ± SEM. Data were analyzed with a two tailed Student's *t*-test using GraphPad Prism 4 (GraphPad Software Inc., La Jolla, CA, USA). Statistical significance was accepted at the level $p < 0.05$.

## RESULTS

Signal for 5-$HT_{1A}$ receptor mRNA was detected in the amygdala (**Figures 1D,E**), DRN (**Figures 2B,C**), and hippocampus (**Figures 3E,F**), and for SERT mRNA in the DRN (**Figures 4B,C**). The level of staining in each case allowed densitometric analysis. Negative controls were performed using an excess of unlabelled cDNA probe during the hybridisation stage (not shown).

### 5-$HT_{1A}$ RECEPTOR mRNA EXPRESSION

Early-life stress significantly increased the levels of 5-$HT_{1A}$ receptor mRNA in the basomedial amygdala (BMA; **Figure 1A**; NS vs. MS: 10.39 ± 0.49 vs. 13.58 ± 0.36; $t(10) = 5.226$, $p < 0.001$), basolateral amygdala (BLA; **Figure 1B**; NS vs. MS: 4.27 ± 0.18 vs. 5.55 ± 0.33; $t(10) = 3.373$, $p < 0.01$) and central amygdala (CeA; **Figure 1C**; NS vs. MS: 3.35 ± 0.14 vs. 4.86 ± 0.51; $t(10) = 2.838$, $p < 0.05$), and decreased the expression of this transcript in the DRN (**Figure 2A**; NS vs. MS: 25.85 ± 1.49 vs. 18.87 ± 1.1; $t(10) = 3.804$, $p < 0.01$) when compared to NS controls. However, densitometric analysis of the 5-$HT_{1A}$ receptor transcript revealed no differences between MS and NS animals in any of the hippocampal layers (**Figure 3**).

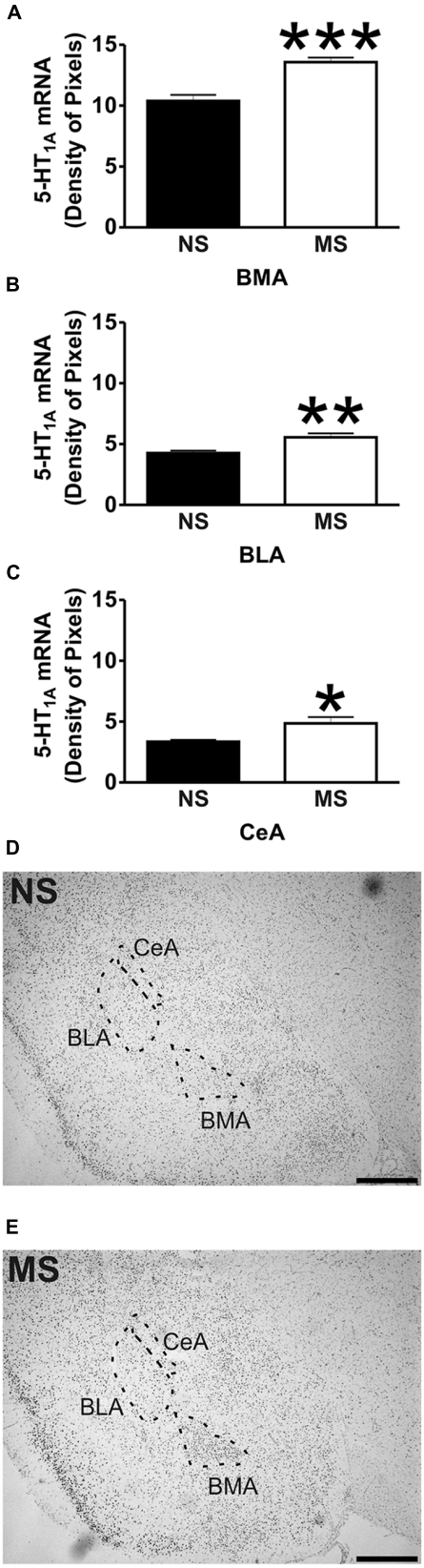


**FIGURE 1 | Continued**

**FIGURE 1 | Continued**
**5-HT$_{1A}$ receptor mRNA expression in the amygdala.** Maternal separation (MS) increases 5-HT$_{1A}$ receptor mRNA expression in three different areas of the amygdala in comparison to non-separated (NS) animals. Graphical representations of the densitometric analysis in the basomedial amygdala (BMA; ***$p < 0.001$; **A**), basolateral amygdala (BLA; **$p < 0.01$; **B**), and central amygdala (CeA; *$p < 0.05$; **C**). Representative microphotographs of 5-HT$_{1A}$ receptor mRNA expression in NS **(D)** and MS **(E)** animals (scale bar represents 1 mm; NS, $n = 6$ and MS, $n = 6$).

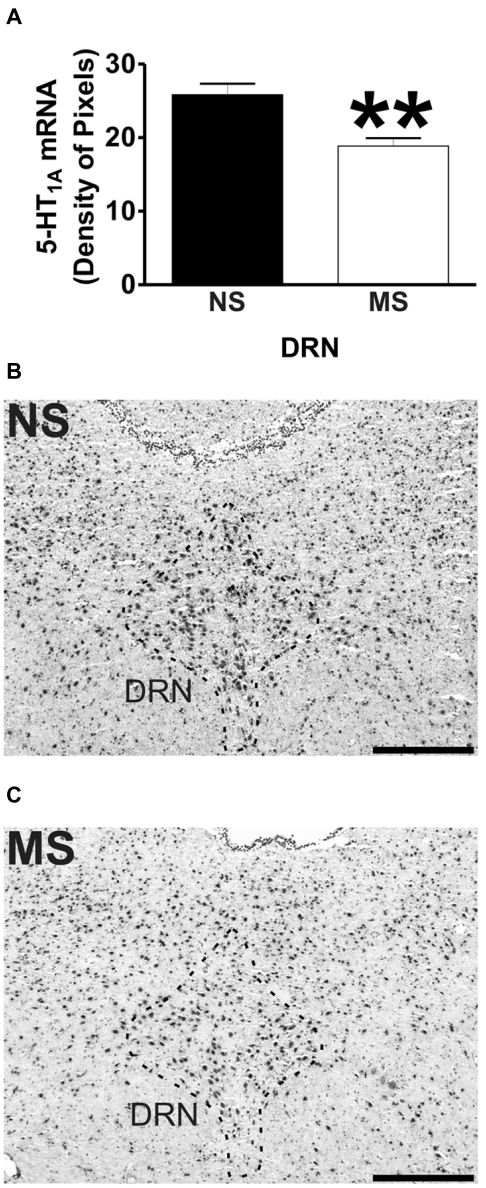


**FIGURE 2 | 5-HT$_{1A}$ receptor mRNA expression in the DRN.** Maternal separation (MS) reduces 5-HT$_{1A}$ receptor mRNA expression in the DRN in comparison to NS animals. Graphical representations of the densitometric analysis in the DRN (**$p<0.01$; **A**). Representative microphotographs of 5-HT$_{1A}$ receptor mRNA expression in NS **(B)** and MS **(C)** animals (scale bar represents 500 μm; NS, $n = 6$ and MS, $n = 6$).

### SERT mRNA EXPRESSION

Maternal separation induced a significant reduction to the transcript for SERT in the DRN in comparison to NS rats (**Figure 4A**; NS vs. MS: $7.58 \pm 0.43$ vs. $5.38 \pm 0.37$; $t(10) = 3.840$, $p < 0.01$).

## DISCUSSION

The present data shows that early-life stress affects gene expression in adulthood, contributing to inadequate stress responses and could therefore lead to the manifestation of stress-related psychiatric disorders. Similar alterations have been described for another neurotransmitter system (Bravo et al., 2010), which further suggest that early-life stress does affects CNS function. Although MS did not affect 5-HT$_{1A}$ receptor mRNA expression in the hippocampus, a structure involved in memory and learning (Jacobson and Sapolsky, 1991; Vizi and Kiss, 1998), it increased its expression in three subregions of the amygdala, a structure related to the control of emotions and fear (LeDoux, 2007). In addition, MS reduced 5-HT$_{1A}$ receptor mRNA expression in the DRN, the major source of serotonergic input to the forebrain which is involved in the control of the central serotonergic tone (Graeff et al., 1996). Also, early-life stress reduced the expression of SERT mRNA in the DRN, which could have an impact on the bioavailability of 5-HT in projection areas of the DRN. These alterations suggest that the behavioral, physiological and molecular deficits described for this animal model (Ladd et al., 1996; Schmidt et al., 2004; Lippmann et al., 2007; O'Mahony et al., 2009) could arise as a consequence of changes in gene expression in key brain regions involved in the development of stress-related psychiatric disorders.

Early-life stress, such as that induced by MS, physical, sexual and emotional abuse and general neglect during childhood, has been associated with serious psychiatric impairments in adulthood (MacMillan et al., 2001; Lupien et al., 2009). During postnatal development the brain undergoes a variety of adaptive changes that depend mostly on the type of stimuli being received (Schmidt et al., 2004). In rats there is a period of reduced stress responsiveness during the first 2 weeks of life (Sapolsky and Meaney, 1986) which can be disinhibited by prolonged MS (Schmidt et al., 2004; O'Mahony et al., 2009). These long periods of MS immediately impact brain gene expression, including the 5-HT$_{1A}$ receptor. For example, Goodfellow et al. (2009) show that 5-HT$_{1A}$ electrophysiological activity in the prefrontal cortex is enhanced in the first 2 to 3 postnatal weeks after MS (3 h a day from P2 to P14). Moreover, mRNA expression of the 5-HT$_{1A}$ receptor in maternally separated animals is increased at postnatal day 9 (Goodfellow et al., 2009). However, when these animals reach adulthood (≥P40), the electrophysiological effects mediated by the 5-HT$_{1A}$ receptor are not different between maternally separated rats and their respective controls, and there is no difference in mRNA expression between rats subjected to early-life stress and control animals (Goodfellow et al., 2009). Nevertheless, exposure to social isolation stress reduces the 5-HT$_{1A}$-elicited electrophysiological activity in the prefrontal cortex of animals that were exposed to early-life stress, in comparison to control rats (Goodfellow et al., 2009), thus suggesting that early-life stress increases the susceptibility toward stress-related psychiatric disorders in adulthood.

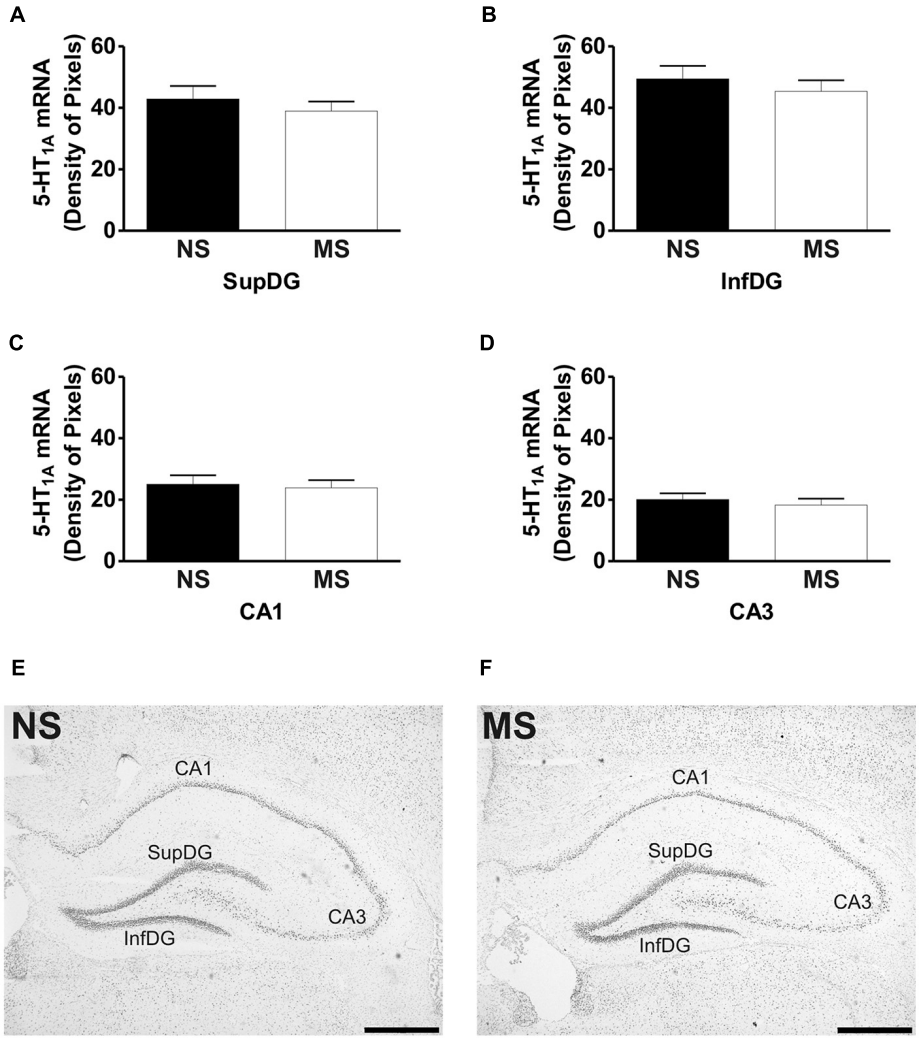


**FIGURE 3 | Hippocampal expression of 5-HT$_{1A}$ receptor mRNA.** Maternal separation (MS) did not affect 5-HT$_{1A}$ receptor mRNA expression in the hippocampus when compared to NS animals. Graphical representations of the densitometric analysis in the suprapyramidal layer of the dentate gyrus (SupDG; **A**), infrapyramidal layer of the dentate gyrus (InfDG; **B**), cornus ammon field 1 (CA1; **C**) and cornus ammon field 3 (CA3; **D**). Representative microphotographs of 5-HT$_{1A}$ receptor mRNA expression in NS **(E)** and MS **(F)** animals (scale bar represents 1 mm; NS, $n = 6$ and MS, $n = 6$).

We have previously described alterations in serotonin metabolism in MS rats (O'Mahony et al., 2008), and additionally, alterations in the central serotonergic system have also been described as a result of different MS procedures (Arborelius and Eklund, 2007; Oreland et al., 2009). Oreland et al. (2009) have shown that brief exposures to MS (15 min from P1 to P13) reduces brain stem expression of 5-HT$_{1A}$ receptor mRNA (Oreland et al., 2009), a procedure that can also affect other neurotransmitter systems (Jaworski et al., 2005; Plotsky et al., 2005). This type of MS could be considered a more naturalistic stress as it mimics the natural rearing environment of rats, where the mother leaves her pups for short periods to forage (Arborelius et al., 2004; Arborelius and Eklund, 2007; Oreland et al., 2009). On the other hand, there is also evidence demonstrating that brief and long daily periods of MS do not affect 5-HT$_{1A}$ receptor and SERT mRNA expression (Arborelius et al., 2004), and furthermore, it has been shown that long periods of daily MS are more effective in producing changes in behavior and alterations in biomarkers associated to stress-related psychiatric disorders (Lippmann et al., 2007; O'Mahony et al., 2009; Bravo et al., 2010). Our previous studies demonstrated that a protocol consisting of 3 h of MS from P2 to P12 produces an increase in corticosterone levels (O'Mahony et al., 2009) and an increase in serotonin turnover (O'Mahony et al., 2008). However, quantitative real time PCR (qRT-PCR) revealed no differences in the expression of 5-HT$_{1A}$ receptor and SERT transcripts in complete brainstem homogenates of MS animals in comparison to NS rats (O'Mahony et al., 2008). Whilst qRT-PCR is a sensitive technique used to assess gene expression, is also a crude method which dilutes any localized changes in gene expression that might occur as a result of early-life stress. Therefore, the present findings corroborate that alterations in serotonin metabolism, induced by MS (O'Mahony et al., 2008), can be consequence of changes in

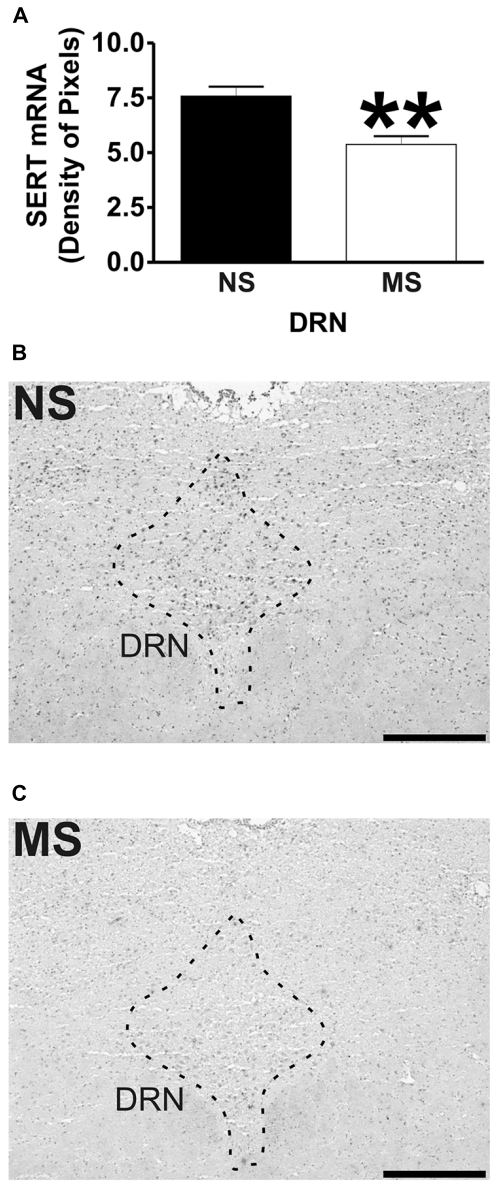


**FIGURE 4 | SERT mRNA expression in the DRN.** Maternal separation (MS) reduces SERT mRNA expression in the DRN in comparison to NS animals. Graphical representations of the densitometric analysis in the DRN (**$p < 0.01$; **A**). Representative microphotographs of SERT mRNA expression in NS **(B)** and MS **(C)** animals (scale bar represents 500 μm; NS, $n = 6$ and MS, $n = 6$).

gene expression within the DRN. However, it is important to note that the present observations only represent changes at the mRNA level and not protein, and they could be just a reflection of a more complex situation involving other neurotransmitter systems (Bravo et al., 2010) and a variety of intracellular cascades that can affect the expression of these transcripts in the different studied areas.

Maternal separation affected the expression of 5-$HT_{1A}$ receptor mRNA in the amygdala. The transcript for this receptor has been described in the rat BMA (Chalmers and Watson, 1991; Pompeiano et al., 1992), and binding of radio labeled 5-$HT_{1A}$ receptor antagonists has been shown in the BLA and CeA (Vicentic et al., 2006). In the present study, early-life stress increased the levels of the transcript for 5-$HT_{1A}$ receptor in the BMA, and also in the BLA and CeA, although in these areas the level of transcript was much lower than in the BMA. It has been shown that activation of 5-$HT_{1A}$ receptors within the amygdala using the agonist 8–Hydroxy-2-(dipropylamino)tetralin (8-OH-DPAT) reduces the levels of social interaction of male rats (Gonzalez et al., 1996), demonstrating that 5-$HT_{1A}$ activation in the amygdala of rats mediates anxiogenic effects. Moreover, and in line with the present findings, Vicentic et al. (2006) showed that non-handled rats (similar to our NS condition) have lower binding capacity of the 5-$HT_{1A}$ receptor antagonist 4-(2′-methoxyphenyl)-1-[2′-[*N*-(2′′-pyridinyl)-*p*-iodobenzamido] ethylpiperazine (pMPPI) in the BMA and BLA in comparison to MS animals. Therefore, the increase in 5-$HT_{1A}$ receptor mRNA expression within the amygdala of MS rats could account for some of the behavioral changes observed by Lippmann et al. (2007), where MS reduced locomotor activity, increased acoustic startle and also affects HPA axis responsiveness in adulthood (Ladd et al., 2005; Lippmann et al., 2007; O'Mahony et al., 2009).

In contrast to the increased expression of 5-$HT_{1A}$ receptor mRNA in the amygdala, there were lower levels of 5-$HT_{1A}$ receptor mRNA found in the DRN of MS rats in comparison to their controls. In this structure there is a high density of 5-$HT_{1A}$ receptors (Blier and de Montigny, 1987; Hutson et al., 1989; Hjorth and Sharp, 1991; Kreiss and Lucki, 1994; Cryan et al., 2005; Savitz et al., 2009), and activation of these presynaptically located receptors decreases the firing frequency, 5-HT synthesis and release from these neurons (Blier and de Montigny, 1987; Sprouse and Aghajanian, 1987; Hjorth and Magnusson, 1988; Hutson et al., 1989; Sharp et al., 1989; Kreiss and Lucki, 1994; Cryan et al., 2005). In addition, 5-$HT_{1A}$ receptor activation in the DRN has been shown to produce anxiolytic effects in different animal models (Andrews et al., 1994; Hogg and File, 1994; Jolas et al., 1995; Picazo et al., 1995; File et al., 1996; Remy et al., 1996; Romaniuk et al., 2001; Koprowska et al., 2002). The reduced levels of 5-$HT_{1A}$ receptor mRNA in the DRN of MS rats suggests an impaired regulation of the central serotonergic tone that could translate into inadequate behaviors toward stressful situations such as those observed by Lippmann et al. (2007). Moreover, we have previously shown that MS rats have altered 5-HT and 5-HIAA levels in the brain stem that clearly suggests an increased turnover of this neurotransmitter (O'Mahony et al., 2008). This could be a consequence of the lower 5-$HT_{1A}$ receptor expression in the DRN, as a lower level of this receptor could impact on the frequency of discharge and/or synthesis and release of 5-HT and therefore affect the neurotransmitter's metabolism. In line with the previous suggestion, Ase et al. (2000) showed that 5-$HT_{1A}$ receptor knock-out mice have increased 5-HT turnover. However, these animals show no differences in basal levels of 5-HT in forebrain areas (Ase et al., 2000; He et al., 2001; Knobelman et al., 2001) and in the DRN (Ase et al., 2000; Bortolozzi et al., 2004) in comparison to wild-type controls. These observations argue against a role of presynaptic 5-$HT_{1A}$ receptors in the maintenance of the central serotonergic tone,

and therefore reveal the complexity of 5-HT neurotransmission regulation.

Another level of regulation to the central 5-HT neurotransmission involves SERT. The levels of SERT mRNA were lower in the DRN of MS rats in comparison to NS rats, which suggest that the reuptake of the neurotransmitter could be affected. The importance of this finding is that changes in SERT expression have been related to psychiatric disorders. For instance, during treatment with SSRIs, the most widely prescribed antidepressants (Frazer, 1997), SERT gets downregulated, which seems to correlate with the efficacy of the treatment (Benmansour et al., 1999, 2002; Frazer and Benmansour, 2002; Gould et al., 2003; Thakker et al., 2004, 2005). However, SERT deficient mice display anxiety- and depression-like behaviors (Holmes et al., 2002; Lira et al., 2003), which suggest that the absence of this gene from early developmental stages affects the ability to cope with stressful situations throughout life. In addition, animals treated during early development with SSRIs also display altered behaviors in adulthood (Mirmiran et al., 1981; Vogel et al., 1990), as the antidepressant would down regulate SERT in early-life. Moreover, downregulation of SERT in adult animals resembles the effects of antidepressant treatment (Thakker et al., 2004, 2005), further highlighting an important role of SERT in the development of the serotonergic system during early-life. In the present study, the reduction in SERT mRNA expression in the DRN not only could affect local serotonin levels (and perhaps its turnover), but it could also impact the adequate development of the serotonergic system and therefore affect the ability to cope with stress. In addition, the reduction in SERT mRNA indicates an alteration that is in line with clinical findings. Individuals with a short allele for SERT, that reduce the efficiency of the gene's transcription, showed more depressive symptoms in relation to stressful events than individuals with the long version of the allele (Caspi et al., 2003), and therefore are at a higher risk of developing psychiatric disorders such as depression.

In summary, the present findings, along with previous observations on other neuronal systems (Bravo et al., 2010) strongly suggest that early-life stress permanently affects gene expression in the CNS. These changes in 5-$HT_{1A}$ receptor and SERT mRNA reflect alterations in a neurotransmitter system that has been extensively related to the development of mood disorders. Moreover, these changes were observed in key brain areas related to the behavioral response to stress. Therefore, these data helps to understand how early-life stress contributes to the development of mood disorders later in life.

## AUTHOR CONTRIBUTIONS

Javier A. Bravo, Timothy G. Dinan and John F. Cryan designed research; Javier A. Bravo performed research and acquired data; Javier A. Bravo, Timothy G. Dinan and John F. Cryan interpreted and analyzed data; and Javier A. Bravo, Timothy G. Dinan and John F. Cryan drafted, revised and wrote the paper.

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REVIEW
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# Alteration of somatosensory response in adulthood by early life stress

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Early life stress is well-known as a critical risk factor for mental and cognitive disorders in adulthood. Such disorders are accompanied by altered neuro- (synapto-) genesis and gene expression. Because psychosomatic disorders induced by early life stress (e.g., physical and/or sexual abuse, and neglect) have become a socio-economic problem, it is very important to clarify the mechanisms underlying these changes. However, despite of intensive clinical and animal studies, such mechanisms have not yet been clarified. Although the disturbance of glucocorticoid and glutamate homeostasis by stress has been well-documented, it has not yet been clarified whether such disturbance by early life stress persists for life. Furthermore, since previous studies have focused on the detection of changes in specific brain regions, such as the hippocampus and prefrontal cortex, it has not been clarified whether early life stress induced changes in the sensory/motor system. Thus, in this review, we introduce recent studies on functional/structural changes in the somatosensory cortex induced by early life stress. We believe that this review provides new insights into the functional alteration of the somatosensory system induced by early life stress. Such information may have clinical relevance in terms of providing effective therapeutic interventions to early life stressed individuals.

**Keywords: maternal deprivation, *in vivo* imaging, *in vivo* microdialysis, glutamate reseptor, spine**

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## Introduction

Early life stress during the perinatal period induces functional and anatomical changes in the brain. Unfortunately, some of such changes persist in adulthood. Clinical studies have shown that early life stress during childhood persistently impairs cognitive and emotional functions, sometimes until adulthood (Chugani et al., 2001; Nelson et al., 2007; Gatt et al., 2009; Mueller et al., 2010; Gershon et al., 2013; Pesonen et al., 2013). Stress-induced alterations of neuronal activity and stress-related hormone secretion may affect neurological development such as dendrite arborization, synaptogenesis, and spine formation (McEwen, 1999; Vyas et al., 2002; Liston et al., 2006). These perinatal stress-induced morphological changes may alter the brain function throughout life (Romeo and McEwen, 2006; Shair, 2007; Gershon et al., 2013). However, it has not yet been clarified how the type, intensity and duration of stress affect different brain regions with different persistence (Romeo and McEwen, 2006). For example, although it has been well-known since several decades ago that acute stress disturbs glutamate and/or corticosterone homeostasis (Moghaddam, 1993; Moghaddam et al., 1994), it has not yet

been clarified whether such a glutamate/corticosterone disturbance persists for a long time in a region- and temporal-specific manner after early life stress exposure (Popoli et al., 2011).

To study the effect of early life stress, various animal models have been introduced with their potential applications in humans (Shair, 2007). In these models, early life stress induces various disorders in adulthood, e.g., an enhancement of anxiety-related behaviors (Wigger and Neumann, 1999; Parfitt et al., 2004; Slotten et al., 2006; Shair, 2007). Such behavioral alteration is partly induced by structural changes of hippocampal neurons and changes in the rate of release of several neurotransmitters/hormones in this region (Brunson et al., 2005; Aisa et al., 2007; Oomen et al., 2010). The electrophysiological changes are also detected in the hippocampus of aged animals (Sousa et al., 2014). Early life stressed rodents also show neuronal changes such as those in synaptic spine density in the infralimbic cortex (Ovtscharoff and Braun, 2001). Early life stress also affects the expression levels of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor by suppressing the function of $Ca^{2+}$ calcium/calmodulin-dependent protein kinase type II (CaMKII) in the barrel cortex (Miyazaki et al., 2012). These findings indicate that early life stress during the perinatal period affects the brain function/structure in various brain regions. However, most studies have been performed focusing in several specific brain regions such as the hippocampus and prefrontal cortex (Wigger and Neumann, 1999; Parfitt et al., 2004; Slotten et al., 2006; Shair, 2007; Miyazaki et al., 2012). Thus, it has not yet been clarified whether early life stress disrupts the somatosensory function.

In this review, we discuss the effect of early life stress on somatosensory function by introducing our recent studies. Most of the results were obtained by *in vivo* studies such as *in vivo* imaging using two- (multi-) photon laser microscopy and free-moving *in vivo* microdialysis. This review may provide novel insights into the functional alterations of the somatosensory system induced by early life stress. Such information may be useful in terms of providing effective therapeutic interventions to early life stressed individuals.

## Persistent Alteration of Synaptic Turnover in the Somatosensory Cortex in Early Life Stressed Mice

Effects of early life stress can be seen in various brain regions. Such effects are observed as changes in synaptic spine density, synaptic turnover rate, electrophysiological properties, neurotransmitters release or expression levels of neuronal and glial proteins. However, it is difficult to identify specific regions affected by a specific early life stress. Even if a particular neuropsychological phenotype is observed, it may still be difficult to identify the affected brain regions, because of the complexity of the mechanisms underlying these changes. In the hippocampus, for example, early life stress alters the spine density and dendritic outgrowth of pyramidal neurons (Magarinos and McEwen, 1995; Pawlak et al., 2005; Monroy et al., 2010; Magarinos et al., 2011), with the change in the expression levels of proteins such as neurotrophic factors and transcription factors (Lippmann et al., 2007; Nair et al., 2007; Kawano et al., 2008; Magarinos et al., 2011; Horii-Hayashi et al., 2013; Suri et al., 2013; Dimatelis et al., 2014). Such an alteration may lead to impaired memory acquisition and cognitive function (Huot et al., 2002; Aisa et al., 2007; Fabricius et al., 2008; Suri et al., 2013; Connors et al., 2015). However, involvement of other brain region cannot be excluded. On the other hand, the somatosensory cortex receives sensory information such as pain, temperature, and pressure, integrates them to identify the object (Haggard, 2006). Its disorder produces difficulties in interpreting tactile information (Freund, 2003; Tinazzi et al., 2013). However, the effect of early life stress on somatosensory function has not yet been clarified. In the somatosensory cortex, the spine density is rather stable after early life stress compared with those in the hippocampus not only in juveniles but also in adults (Takatsuru et al., 2009a). Nevertheless, the nociceptive threshold is significantly decreased in early life stressed juvenile (4 weeks-old) and adult mice (12 weeks-old; Takatsuru et al., 2009a). Electrophysiologically, the slope (μV/ms) of field potential in layer II/III evoked by vibrotactile somatosensory simulation increases in early life stressed adult mice. Interestingly, these changes significantly correlate with the decrease in nociceptive threshold (**Figure 1**; Toya et al., 2014), indicating that somatosensory function is persistently altered by early life stress.

As discussed above, although the somatosensory response is altered by the change in electrophysiological properties, previous studies have shown slight morphological changes in the somatosensory cortex (Takatsuru et al., 2009a). To clarify further the mechanism inducing neurological alterations, it is necessary to apply additional techniques such as two-photon laser microscopy (Denk et al., 1990; Grutzendler et al., 2002; Trachtenberg et al., 2002). This technique enables us to examine dynamic changes in synaptic turnover rate and neuronal excitability during neuronal circuit remodeling (Takatsuru et al., 2009b).

In early life stressed mice, the turnover rate of mushroom spines, which usually maintain their structure for a long time (Grutzendler et al., 2002; Trachtenberg et al., 2002), is significantly increased in the somatosensory cortex in not only juvenile but also in adult mice (Takatsuru et al., 2009a). Because both of the gain and loss of spines occur simultaneously, the total number of spines is not markedly altered. However, two-photon microscopy enabled us to detect the persistent dynamic changes in synaptic turnover induced by early life stress. These findings indicate that early life stress destabilizes synaptic formation in the somatosensory cortex, resulting in the disturbance of somatosensory function.

Although we have shown the persistent increase in synaptic turnover rate in the somatosensory cortex induced by early life stress, the mechanisms underlying such an alteration have not yet been clarified. One possibility is the involvement of microglia. Recently, we have found the alteration of motility of microglia in early life stressed mice *in vivo* (Takatsuru et al., 2015). The motility of the filopodia-like processes is increased in early life stressed mice. Interestingly, the motility of the

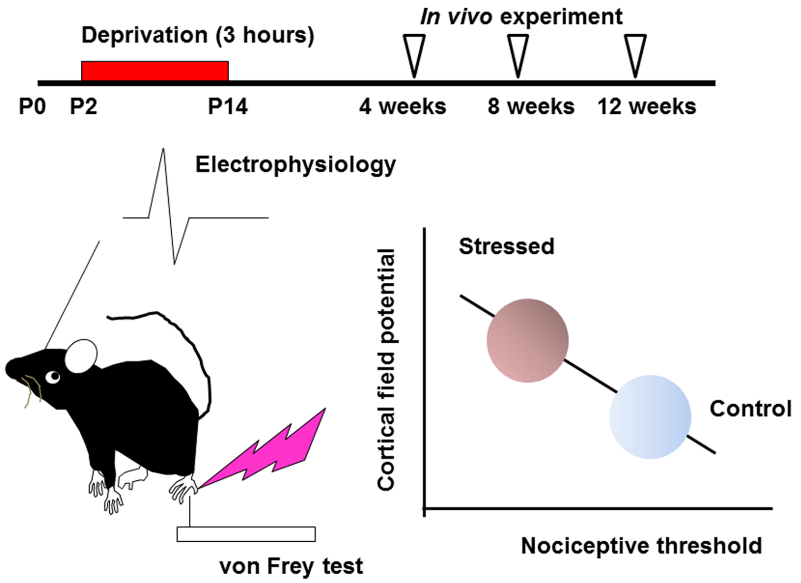


**FIGURE 1 | Early life stress induces hyperactivity of somatosensory function (see Takatsuru et al., 2009a and Toya et al., 2014, for details).** Maternal deprivation is performed from postnatal day P2 to P14, 3 h per day (the day of birth is determined as P0). After weaning, an *in vivo* experiment is performed from 4 to 12 weeks. In early life stressed mice, the nociceptive threshold studied by von Frey hair test decreases with increasing cortical field potential. These values significantly correlate.

processes negatively correlates with the somatosensory threshold (the motility is higher in the mice with a lower threshold). Furthermore, the number of processes is significantly increased in early life stressed mice after acute somatosensory stimulation and such an increase persists for several hours. The motility of microglia is changed by neuronal conditions as in the case of remodeling of synapses after a focal stroke (Wake et al., 2013). Because previous studies indicate that microglia may partly regulate synaptic formation by removing or 'stripping' synapses (Marín-Teva et al., 2004; Cullheim and Thams, 2007; Tremblay, 2011) and because the direct contact of microglial processes with spines has been observed (Wake et al., 2013), the activity of microglia increased by early life stress may contribute to the structural instability of spines in the somatosensory cortex. In a series of study, we have clarified that not only severe brain damage such as ischemia or inflammation, but also psychological stress such as maternal separation can produce persistent changes in microglial activity. However, early life stress factors activating microglia have not yet been clarified. In the next section, we will discuss several possible factors for such activation.

## Persistent Alterations of Glutamate and Glucocorticoid Homeostasis in the Somatosensory Cortex Induced by Early Life Stress

Early life stress may induce alterations of neurotransmitters (Barbosa Neto et al., 2012; Martisova et al., 2012; Gunn et al., 2013) and neurotrophic factors in various brain regions (Lippmann et al., 2007; Nair et al., 2007; Kawano et al., 2008; Magarinos et al., 2011; Horii-Hayashi et al., 2013; Suri et al., 2013; Dimatelis et al., 2014). Such alteration may produce changes in the structure of neural circuits, spine turnover rate, and/or microglial motility. Since many critical developmental events occur in the neonatal period, even weak environmental insults may produce irreversible alterations in organ homeostasis. Indeed, disruption of homeostasis of glucocorticoid release by administering excess amounts of corticosterone produces various abnormalities such as those in the number of spines, synaptic turnover rate, microglial motility, electrophysiological properties, and astrocyte function (Gunn et al., 2013). However, it has not yet been clarified whether such alterations occur in the somatosensory cortex. Detecting the concentration of a neurotransmitter such as glutamate or GABA in the cortex may reveal the mechanisms underlying the change in the turnover rate of mushroom spines. *In vivo* microdialysis is a potent technique to observe the neuronal transmitter release under intact condition although the spatial resolution is limited. This technique also enables local drug application, which is usually difficult due to the blood–brain barrier (Takatsuru et al., 2013). Using this technique, we have found that the concentration of glutamate is increased in early life stressed mice under free-moving condition (**Figure 2**; Toya et al., 2013, 2014). We have also reported that the homeostasis of coritcosterone is also affected by early life stress (Toya et al., 2014).

Corticosterone may play a protective role in neuronal circuits against stress (McEwen, 2000a,b). Glucocorticoids convert proteins and/or lipids into carbohydrates, which can be easily used for energy production. This conversion will serve the body well in the short run by replenishing energy reserves after a period of activity, as in a situation such as running away from

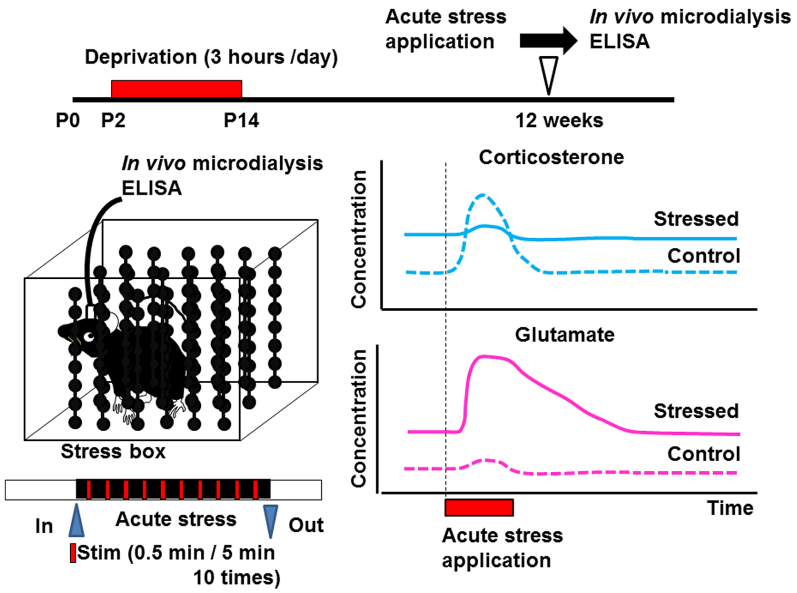


**FIGURE 2 | Schematic drawing showing the effect of early life stress on acute-stress-induced corticosterone and glutamate responses in the somatosensory cortex.** Samples from the somatosensory cortex were collected by *in vivo* microdialysis. After collecting the dialysate in the home cage, the mice were placed inside the stress box. Stressful physical stimulation (30 s duration, every 5 min) were applied 10 times. The concentrations of corticosterone and glutamate were determined by ELISA and HPLC, respectively, (Toya et al., 2014). Early life stress induced an increase in basal levels of corticosterone and glutamate. On the other hand, although acute-stress-mediated increase in corticosterone concentration did not occur in early life stressed mice, that in glutamate concentration was markedly enhanced.

a predator (McEwen, 2000b). Glucocorticoids also increase the appetite for food and promote food-seeking behavior (Leibowitz and Hoebel, 1997). Thus, enhancement of corticosterone release under acute stress potentially protects the body from stress. However, a long-term increase in corticosterone concentration induces dendritic atrophy in some brain regions (McEwen,

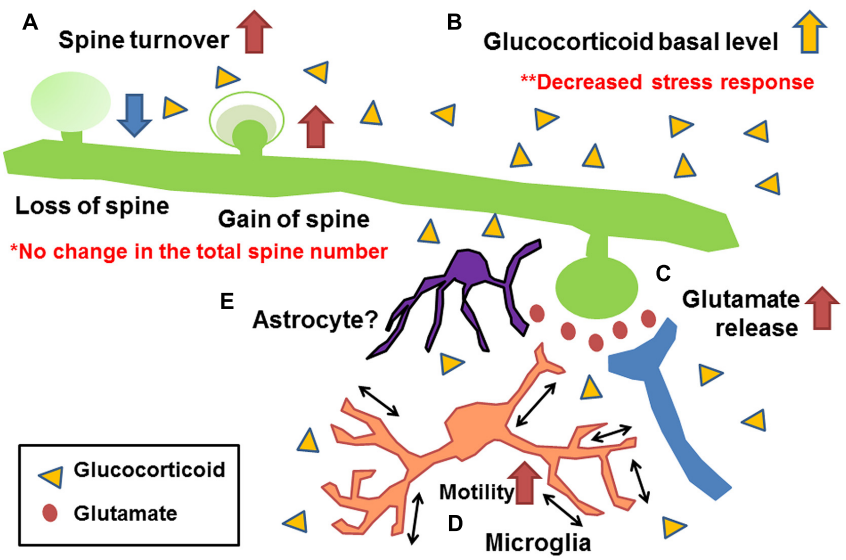


**FIGURE 3 | Schematic drawing showing the morphological/chemical alterations induced by early life stress in the somatosensory cortex (Takatsuru et al., 2009a, 2015; Toya et al., 2014). (A)** Increase in mushroom spine turnover rate without significant alteration of total number of spines, **(B)** increase in basal glutamate level and acute-stress-mediated increase in glutamate level, **(C)** increase in basal level of corticosterone without further increase under acute stress, and **(D)** increase in microglial motility. Although the involvement of astrocytes has not yet been clarified **(E)**, studies of other brain regions indicate their possible involvement (Gunn et al., 2013).

2000a) and thus, the concentration of corticosterone should be carefully controlled to maintain the homeostasis of body/brain functions. Furthermore, perinatal stress sometimes disrupts hypothalamic-pituitary-adrenal axis, resulting into persistent aberrant glucocorticoid secretion (Faturi et al., 2010; Koe et al., 2014; Nishi et al., 2014). Under such conditions, the protective role of glucocorticoids in neural circuits may be disrupted. Thus, an increased basal corticosterone concentration in the somatosensory cortex in early life stressed animals may be partly involved in inducing spine instability, glutamate secretion, and microglia motility. Disruption of glucocorticoid responses to acute stress may exacerbate such instability.

In early life stressed animals, the basal concentration of glutamate in the somatosensory cortex increases markedly (Toya et al., 2014). It has been well-known that the acute stress disrupts glutamate homeostasis (Moghaddam, 1993). However, it has not yet been clarified whether such disruption persists for life after early life stress in a specific brain region. Our study clearly demonstrated the increase in glutamate level in adulthood by early life stress in the somatosensory cortex. On the other hand, in control mice, the concentration of glutamate is rather stable after acute-stress application (Toya et al., 2014). This is probably due to the activation of glial cells that take up excess glutamate, thus preventing excitotoxity (Danbolt, 2001; Takayasu et al., 2009). In early life stressed mice, on the other hand, although the basal glutamate concentration is sixfold that in control mice, acute stress further increased the concentration of glutamate. Such a further increase lasts longer than 1 h after stimulation. These findings indicate the alteration of glutamate homeostasis by early life stress. However, the interaction between enhanced glutamate release and suppressed corticosterone response induced by acute stress early in life of mice has not yet been clarified.

Although the mechanisms inducing the persistently enhanced glutamate release in the somatosensory cortex induced by early life stress have not yet been fully understood, previous studies have provided several clues. Environmental stress enhances glutamate release and suppresses glial-cell-mediated glutamate cycling. Such changes affect synaptic transmission in the limbic/cortical areas (Sanacora et al., 2012). Early life stress also affects the structural organization, i.e., dendritic remodeling, reduction of synaptic spine formation, glial cell loss, and possibly volumetric reductions of several specific brain regions of the rodent brain (Sanacora et al., 2012). Acute exposure to stress or administration of glucocorticoids rapidly promotes glutamate release in the hippocampus and other brain regions (Lowy et al., 1993; Moghaddam, 1993; Reznikov et al., 2007). The glucocorticoid-receptor-mediated increase in the expression levels of presynaptic soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complexes are induced in the presynaptic membrane in the prefrontal/frontal cortex by acute stress. SNARE then enhances the release of glutamate (Musazzi et al., 2010). The Rab4-mediated recycling of NMDA and AMPA receptors from early endosomes is also enhanced in the prefrontal cortex (Yuen et al., 2009). Chronic stress also decreases the number of glial fibrillary acid protein (GFAP)-expressing cells and the impaired clearance of synaptic glutamate through excitatory amino acid transporters in the prefrontal cortex (Banasr and Duman, 2008). Taken together, it is reasonable to speculate that early life stress may alter glucocorticoid homeostasis, and such an alteration may enhance glutamate release by stimulating the expression of proteins related to glutamate release and sensitivity. The activity of glial cells, which take up glutamate, may also be affected, inducing an increase in interstitial glutamate level.

On the basis of the findings mentioned above, we examined the involvement of glutamate receptor subunits of AMPA, NMDA, and metabotropic glutamate receptors after application of acute stress in early life stressed mice to clarify the underling mechanisms by Western blot analysis (Toya et al., 2013). However, protein levels of these subunits in the membrane fraction were not significantly different between the control and early life stressed animals before and after acute-stress application. Because we carried out only Western blot analysis, further study to determine the protein turnover rate by *in vivo* imaging may be required. Under the present condition, however, we were unable to detect the involvement of glutamate receptors in the alteration of somatosensory function induced by early life stress.

## Summary and Perspectives

**Figure 3** shows a summary of the effects of early life stress in the somatosensory cortex. We found the following; (A) Increase in mushroom spine turnover rate without significant alteration of the total number of spines, (B) Increase in basal level of corticosterone without further increase under acute stress, (C) Increase in basal and acute-stress-mediated glutamate levels, and (D) Increase in microglial motility. A combination of these alterations may have affected somatosensory function. Unfortunately, however, we failed to identify molecules involved in such alterations. One possible reason for the failure is that, although we detected a significant increase in synaptic turnover rate and microglial motility with a decreased somatosensory threshold and an increased electrophysiological activity, less than 10% of all spines were lost or gained. Thus, only a limited amount of molecules may be involved in such subtle changes. To detect such changes, Western blot analysis may not be a suitable technique. More sophisticated techniques such as detection of protein trafficking and/or protein expression at the single-cell level may be required. Nevertheless, our series of studies have demonstrated the persistent alteration of somatosensory function induced by early life stress with morphological/chemical alterations in the somatosensory cortex. Although the involvement of astrocytes has not yet been clarified, a previous study has shown that early life stress decreases glutamate uptake in the hypothalamus (Gunn et al., 2013). Thus, such a decrease may also be induced in the somatosensory cortex (**Figure 3E**). Trials to clarify the involvement of astrocytes are currently underway. However, as discussed above, the morphological/chemical changes in the somatosensory cortex are not always identical to those in other brain regions. Thus, more

careful analysis may be required to examine the brain region-specific effects of early life stress. In particularly, more attention should be paid to the change in somatosensory function in early life stressed humans, because only limited information is available at present. We believe that this review has provided an important clue to developing effective therapeutic interventions to prevent persistent somatosensory abnormalities induced by early life stress.

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