Epigenetische Einflüsse während der Entwicklung und deren neurobiologische Konsequenzen bei psychiatrischen Erkrankungen

Dissertation zur Erlangung des Grades
eines Doktors der Naturwissenschaften
der Fakultät für Biologie und Biotechnologie
der Ruhr-Universität Bochum

Internationale Graduiertenschule Biowissenschaften
Ruhr-Universität Bochum
Experimentelle und molekulare Psychiatrie

Vorgelegt von
Annakarina Mundorf

aus
Bergisch Gladbach,

Bochum
Juli, 2020

Referent: Jun.-Prof. Dr. Nadja Freund
Korreferent: Prof. Dr. Stefan Herlitze

Epigenetic influences during development and their neurobiological consequences in psychiatric disorders

Dissertation to obtain the degree
Doctor Rerum Naturalium (Dr. rer. nat)
at the Faculty of Biology and Biotechnology
Ruhr University Bochum

International Graduate School of Bioscience
Ruhr University Bochum
Experimental and Molecular Psychiatry

submitted by
Annakarina Mundorf

from
Bergisch Gladbach, Germany

Bochum
July, 2020

First supervisor: Jun.-Prof. Dr. Nadja Freund
Second supervisor: Prof. Dr. Stefan Herlitze

Table of Contents

List of Figures

Figure permissions:

Figure 2: Reprinted by permission from Springer Nature: Nature Rev, Effects of stress throughout the lifespan on the brain, behaviour and cognition, Lupien SJ, McEwen BS, Gunnar MR, Heim C, Copyright 2009

Figure 3: Reprinted by permission from Springer Nature: Nature, Unfinished symphony, Jane Qiu, Copyright 2006

List of Abbreviations

ADHD	Attention deficit hyperactivity disorder
ACTH	Adrenocorticotrophic hormone
AVP	Arginine vasopressin
CRH	Corticotropin-releasing hormone
D	Dopamine
ELS	Early life stress
EWAS	Epigenome-wide association studies
fMRI	Functional magnetic resonance imaging
HPA	Hypothalamic–pituitary–adrenal
GC	Glucocorticoid
GR	Glucocorticoid receptor
GWAS	Genome-wide association studies
ISO	Social isolation
MDD	Major depressive disorder
MR	Mineralocorticoid receptor
MRI	Magnetic resonance imaging
MS	Maternal separation
OTXR	Oxytocine receptor gene
PNN	Perineural nets
PTSD	Posttraumatic stress disorder

Zusammenfassung

Epigenetische Modifikationen, darunter DNA-Methylierung und Histon Modifikationen, sind wichtige Einflüsse im Laufe der Gehirnentwicklung, da sie die Genexpression unabhängig vom Genotyp verändern können. Die Gehirnentwicklung zeichnet sich durch verschiedene Phasen des beschleunigten Wachstums und Reifeprozesse aus. Starkes Stress-Erleben in diesen sensitiven Phasen der Gehirnentwicklung führt zu fundamentalen neuronalen Veränderungen, wie z.B. reduziertes Hippocampusvolumen und Hyperreaktivität neuronaler Schaltkreisläufe. Diese Veränderungen führen wiederum zu der Entstehung von psychiatrischen Erkrankungen. Stress als ein Umwelteinfluss, kann epigenetische Muster verändern und so zu schädlicher Hemmung der Expression oder auch Überexpression bestimmter Gene führen. Folglich können epigenetische Modifikationen in sensitiven Phasen der Gehirnentwicklung zwischen Umwelteinflüssen und induzierten neurobiologischen Konsequenzen mediieren. Das Potential der DNA-Methylierung als Mediator zwischen Umwelteinfluss und Psychopathologie ist vielversprechend, jedoch müssen die neuronalen Korrelate veränderter DNA-Methylierung zuvor noch identifiziert werden. Tiermodelle zeigen nach frühkindlichem Stress Konsequenzen ähnlich denen im Menschen. Sie ermöglichen daher die Untersuchung von neurobiologischen Veränderungen, während der Gehirnentwicklung, verantwortlich für die resultierenden Beeinträchtigungen. Um die involvierten Mechanismen zu entschlüsseln, müssen verschiedene Ansätze vereint werden. In dieser Arbeit werden daher sowohl Studien mit Menschen als auch am Tier vorgestellt, um mit Hilfe dieser epigenetische Modifikationen im Sinne der DNA-Methylierung und veränderter Genexpression im Kontext der Gehirnentwicklung und Psychopathologie zu untersuchen.

Das Volumen der weißen Substanz steigt im Laufe der Entwicklung stetig an, was die zunehmende Anzahl an neuronalen Verbindungen widerspiegelt. Da Auswirkungen von Stress somit langfristige Beeinträchtigung in der Gehirnentwicklung mit sich ziehen könnten, werden Veränderungen in der weißen Substanz in Zusammenhang mit frühkindlichem Stress und Psychopathologie in dieser Arbeit genauer beleuchtet. In einem translationalen Übersichtsartikel in Kapitel 2 wird die wichtige Rolle von veränderter Integrität der weißen Substanz bei Depression dargestellt. Interessanterweise stellte sich heraus, dass eine Depression einhergehend mit Widrigkeiten in der Kindheit zu anderen Veränderungen führt, als eine Depression ohne Probleme in der Kindheit. Auf dieses Ergebnis aufbauend werden in Kapitel 3 die Folgen von frühkindlichem Stress im Laufe des Lebens auf Verhalten, Neurobiologie und

DNA-Methylierungsmuster untersucht. Ein translationer Übersichtsartikel verdeutlicht, dass Stress in der Kindheit zu langanhaltenden Veränderungen im DNA-Methylierungsmuster führt, welche ebenso in psychiatrischen Krankheiten gefunden werden. Die Experimente an Ratten heben erneut die Kindheit als sensitive Phase für stress-induzierte Beeinträchtigungen hervor. Stress, hervorgerufen durch maternale Separation, führte zu einem Phänotyp, der im Laufe der Entwicklung die Richtung wechselte. Genauer gesagt führte maternale Separation zu weniger Angst in der Kindheit, jedoch zu mehr Angst in der Jugend, während im Erwachsenenalter keine Folgen mehr ersichtlich waren. Dies verdeutlicht die Jugend als besonders sensitive Zeit, in der Veränderungen im Phänotyp am stärksten ausgeprägt sind (atypisches links Drehen und erhöhte Angst). Darüberhinaus zeigte eine Untersuchung der Vokalisation der Ratten, als Maß für den affektiven Zustand, dass jugendliche Ratten deutlich weniger vokalisieren als die anderen beiden Altersgruppen. Die jugendliche Gehirnentwicklung ist durch große hormonelle Veränderungen geprägt, was die Frage aufwirft, ob die postpartale Zeit, ebenso geprägt durch hormonelle Veränderungen, gleichermaßen vulnerabel gegenüber Stress ist. Die Studien in Kapitel 4 zeigen, dass maternale Separation nicht nur die Kinder, sondern auch die Mütter beeinflusst, ähnlich den Veränderungen, die mit der postpartalen Depression bei Menschen einhergehen. So unterstreichen diese Studien die postpartale Zeit als besonders vulnerabel für neuronale Veränderungen.

Im letzten Kapitel werden zwei Kandidatengene, *MORC1* und *GDAP1*, auf ihre spezifische Sensitivität gegenüber einem Umwelteinfluss und entsprechender Psychopathologie genauer untersucht. Hierzu sind zwei Studien mit gesunden Probanden aufgeführt, die erhöhte periphere *MORC1* Methylierung als Indikator für erhöhte depressive Symptome nachweisen. Darüberhinaus konnte in diesen Probanden eine Assoziation von erhöhter peripherer *MORC1* Methylierung und reduzierter hippocampaler Neuritendichte gefunden werden. Die mögliche Anwendung von *GDAP1* Methylierungsmustern, ein vorgeschlagener Biomarker für Alkoholmissbrauch, wurde weitergehend auf seine spezifische Sensitivität überprüft. Hierfür wurde hippocampale GDAP1 Expression in Mäusen untersucht, die zuvor mit Zigarettenrauch beatmet wurden. Da Zigarettenrauch einen regionsabhängigen Effekt auf die Expression von GDAP1 hatte, kann geschlussfolgert werden, dass das *GDAP1* Methylierungsmuster eher ein Indikator für Substanzmissbrauch allgemein ist. Dies verdeutlicht die Schwierigkeit, einen krankheitsspezifischen Marker zu finden. Als letztes wurde die Auswirkungen von wiederholter Lithium Injektion auf die hippocampale Glutamin-Synthetase Promotoraktivität untersucht. Hierfür wurde genetisch veränderten Mäusen wiederholt Lithium oder Natriumchlorid injiziert und diese mit unbehandelten Kontrollen verglichen. Interessanterweise führte wiederholte

Natriumchlorid-Injektion zu erhöhter Promotoraktivität, wohingegen Lithium protektiv wirkte. Drüberhinaus weisen beide Mausstudien daraufhin, dass die Expression von Kandidatengenen als Reaktion auf Umwelteinflüsse verändert wird, vor allem in Regionen von anhaltender Neurogenese. Ebenso schneinen Psychopharmaka eine protektive Wirkung auf die Neurogense zu haben. So ermöglichen die Studien aus Kapitel 5 tiefere Einblicke in das Potential von DNA-Methylierung in der psychiatrischen Diagnostik.

Zusammenfassend unterstreicht diese Arbeit den wichtigen Einfluss von Stress während sensitiven Phasen der Gehirnentwicklung und schlägt epigenetische Modifikationen als Ursache für neuronale Veränderungen vor. Diese Beeinträchtigungen führen dann zur Entstehung von psychiatrischen Krankheiten. Die Studien deuten darüber hinaus auf verschiedene vulnerable Zeitpunkte und in dieser Zeit besonders betroffene Gehirnregionen hin. Die Untersuchung von DNA-Methylierungsmustern in psychiatrischen Erkankungen birgt somit großes Potential, jedoch müssen zunächst noch einige Hürden überwunden werden.

Abstract

Epigenetic modifications, such as DNA methylation and histone modifications, are important in brain development as they enable alterations in gene expression without changing the DNA sequence and thus, allow heterogeneity between genetically homologous cells. Brain development is marked by distinct periods of accelerated growth and maturation. Severe stress exposure during sensitive periods of brain development results in fundamental neuronal impairments, e.g., reduced hippocampal volume or circuit hyper-reactivity. These alterations then lead to psychiatric disorders. Stress as an environmental factor can modify epigenetic patterns leading to harmful silencing or overexpression of certain genes. Therefore, epigenetic modifications during sensitive periods of brain growth are able to mediate between environmental exposure and induced neurobiological implications. The potential of DNA methylation mediating exposure and psychopathology holds promise but sensitive periods and neuronal correlates of altered DNA methylation have to be identified first. Animal models show alterations similar to humans after early life stress, thus allowing for analyzation of neurobiological implications during brain development responsible for impairments. To resolve the complex mechanisms involved, different approaches have to be combined. This thesis, therefore, includes human and rodents studies to disentangle epigenetic modifications in terms of DNA methylation and altered gene expression in the context of brain development and psychopathology.

The white matter volume steadily increases throughout development, reflecting the increasing number in connections and circuitries being formed. As consequences of stress could, therefore, result in longlasting impairments during development, the importance of white matter alterations in association with early adversity and psychopathology are unraveled in this thesis. A comprehensive review of the literature in chapter 2 indicates an important role of altered white matter integrity in depression. Interestingly, alterations on the molecular level found in affected individuals who experienced early adversity were not found in depressive patients without a history of childhood maltreatment. Following this finding, the consequences of early stress exposure throughout life on behavior, neurobiology, and DNA methylation patterns are investigated in chapter 3. First, a comprehensive review highlights that early stress exposure induces longlating alterations in DNA methylation patterns similar to those seen in psychiatric disorders. The experimental studies on rats further underline childhood as a sensitive time for stress-induced impairments. Stress induction by maternal separation resulted in phenotypes

differing in directionality throughout development with juveniles being less anxious after stress exposure and adolescences being most anxious. However, in adults, no lasting effect of exposure were apparent, emphasizing adolescence as the crucial period, in which manifestation of the phenotype (atypical leftward turning and increased anxiety) is most pronounced. Moreover, investigation of ultrasonic vocalization as a measure of affective state revealed reduced communication in adolescence. However, as adolescent brain development is characterized by great hormonal changes in puberty, the question arises whether the postpartum time, which is also marked by great changes in hormonal levels, constitutes a similar vulnerable time for stress-induced impairments. Studies in chapter 4 show that maternal separation not only affects offspring but also results in consequences for dams similar to alterations found in postpartum depression, highlighting the postpartum period as a time of great neuronal vulnerability.

In the last chapter, two candidate genes, namely *MORC1* and *GDAP1* are characterized concerning their sensitivity towards a specific exposure and psychopathology. Included are two research studies with healthy human participants that identified increased peripheral *MORC1* methylation as an indicator of depressive symptoms. Further, imaging techniques allowed to investigate neuronal correlates of peripheral *MORC1* methylation, revealing an association between increased gene methylation and reduced hippocampal neurite density. The potential application of *GDAP1* methylation patterns, a proposed biomarker for alcohol use disorder, was investigated regarding its sensitivity. Therefore, hippocampal GDAP1 protein expression was analyzed in mice previously exposed to cigarette smoke. As cigarette smoke exposure showed region-specific effects on GDAP1 expression, *GDAP1* methylation changes might be more reflective of substance use than solely alcohol use disorder. The study thus highlights the difficulties in identifying disorder-specific candidate genes. Lastly, in a study on genetically modified mice, hippocampal candidate gene activity after chronic injection of either lithium, natrium chloride, or no injection was investigated. These mice allow for the study of glutamine synthetase promotor activity. Interestingly, natrium chloride injection resulted in increased promotor activity whereas lithium injection had protective effects resulting in promotor activity equally to controls. However, both studies in mice indicate, that candidate gene expression is impaired as a response to exposure in regions of ongoing neuronal growth with psychoparmaka potentially acting protective on neurogenesis. Taken together, the studies in chapter 5 allow for deeper insights into a potential psychiatric application of DNA methylation.

To conclude, this thesis underlines the critical impact of stress exposure during sensitive periods of brain development, proposing epigenetic modifications may be causative for neuronal

impairments resulting in psychopathology. Studies included highlight different vulnerable periods and the brain structures implicated during these periods. The study of DNA methylation patterns in psychiatry holds great potential, however, certain challenges have to be faced first.

Chapter 1 |
General Introduction

"All mental processes are brain processes, and therefore all disorders of mental functioning are biological diseases"

Eric R. Kandel, Austrian-American psychiatrist, neuroscientist, and recipient of the 2000 Nobel Prize in Physiology or Medicine

Psychiatric or mental disorders have been described to be disorders of the brain given their main impairment is in mental functioning (American Psychiatric Association, 2013). Even though most psychiatric disorders show high heritability, disentangling their genetic basis has proven to be difficult (Burmeister et al., 2008; Hoehe & Morris-Rosendahl, 2018). One challenge in identifying the genetic basis of disorders is that certain genetic risk factors interact with environmental factors, especially toxic stress, and this interaction then leads to psychopathology (Burmeister et al., 2008). Over the past decades, a new line of rapidly growing research has offered new perspectives on processes mediating between environment and genetic factors: Psychiatric epigenetics (Barker et al., 2017; Cecil et al., 2020). Epigenetics are processes that can reversibly regulate gene activity without altering the DNA sequence itself. Thus, epigenetic modifications, such as DNA methylation, can lead to harmful silencing or overexpression of certain genes causative for certain phenotypes (Mundorf & Freund, 2019). Especially changes in gene expression during sensitive periods of brain development are likely to result in neurobiological impairments causative for psychopathology (Cecil et al., 2020; Meredith, 2015). However, the full impact of neuronal epigenetic regulation on psychiatric disorders is still to be discovered.

In the following chapter, the relevant elements of brain development, psychiatric disorders, and epigenetic processes will be presented. Moreover, the influence of stress exposure on each aspect will be discussed, highlighting its crucial influence on all three topics.

1.1. Brain development from birth to adulthood

In general, human development can be divided into the prenatal period, infancy (birth until the first year), early and late childhood (until puberty), adolescence (begin of puberty), and adulthood (starting in the early twenties) (Lupien et al., 2009). While most of our organs are fully developed until the early postnatal days (e.g., heart, lungs, digestive organs), the brain is not fully developed until late adolescence and does not stop establishing new connections until adulthood (Sadava et al., 2019).

1.1.1. Structural and functional level

Generally, structural brain development in humans is investigated using magnetic resonance imaging (MRI) allowing to investigate gray and white matter changes in volume, thickness, and surface structure (Shaw et al., 2008). The white matter volume constantly increases from childhood to adolescence until adulthood (Brain Development Cooperative Group, 2012; Houston et al., 2014; Koolschijn & Crone, 2013; Paus, 2010; Paus et al., 2001), whereas gray matter volume increases first, peaks around puberty and decreases throughout adolescence and adulthood (Brain Development Cooperative Group, 2012; Giedd et al., 1996; Gogtay et al., 2004; Koolschijn & Crone, 2013; Raznahan et al., 2011; Taki et al., 2013) (**Figure 1)**. Changes in brain volume, thickness, and surface area throughout life have been studied in particular in the hippocampus, amygdala, and prefrontal cortex since these regions develop over a long period (Bramen et al., 2011; Giedd et al., 1996; Gogtay et al., 2004; Lenroot et al., 2007; Raznahan et al., 2011), and are, therefore, appropriate regions when investigating developmental trajectories (Lupien et al., 2009). Throughout development, the hippocampus undergoes a rapid growth in volume from birth until two years of age whereas the amygdala volume increases steadily from birth until the late ’20s (Giedd et al., 1996). The frontal cortex experiences rapid growth in late childhood (8-14 years of age) (Giedd et al., 1996) and, therefore, this is the lastest region to fully establish myelination and synaptic density (Yakovlev et al., 1967).

Besides the developmental stage, sex is another key factor in brain developmental trajectories, especially as sex-specific trajectories are being found. For example, frontal and parietal lobe grey matter volume peaks earlier in girls compared to boys (Lenroot et al., 2007). Subcortical regions such as the hippocampus and amygdala have been found to differ in developmental growth trajectories as well, with sex-differences in volume depending on region and age (Bramen et al., 2011; Neufang et al., 2009). For example, boys show increased volumes in the amygdala, thalamus, putamen, and insula compared to girls (Peper et al., 2009). But the found

sex differences in developmental growth might depend on sexual maturity rather than age, given that sexually more mature adolescents showed greatest sex differences in hippocampus, amygdala and cortical gray matter volume, with mature boys having larger volumes and sexually mature girls revealing smaller volumes (Bramen et al., 2011). However, the investigation of different developmental growth trajectories between age and sex is still in its infancy, especially as studies in adolescents inlcuding individuals with different levels of sexual maturity are still rare but necessary.

Besides structural alterations, brain development has also been investigated with a focus on connectivity and activity. Researchers have thus discovered an individual pattern of brain connections, called the functional connectome (Galván, 2017). Functional connectomes can be assessed when analyzing e.g. functional MRI (fMRI) images during a working memory task, an emotion processing task or while resting (Kaufmann et al., 2017). Interestingly, these connectivity profiles change throughout life peaking in the teenage years when they become more stable and individualized (Kaufmann et al., 2017), thus rendering teenagers more vulnerable to impairments or delays in neurodevelopment. The reward system has been a focus of investigation as well with the striatum (a region implicated in reward processing) being hyper-responsive in adolescents, reflected in greater reward-seeking behavior during this time (Galván, 2010). Another important network experiencing accelerated growth during adolescence is the amygdala-prefrontal cortex circuitry (Tottenham & Galván, 2016). This circuitry develops over a long period until adolescence or even early adulthood and is important for a mature affect regulation (Tottenham & Galván, 2016). Interestingly, despite the prolonged maturation, the amygdala circuitry is most sensitive to impairments during adolescence since its functional connectivity changes from positive connectivity in childhood to a negative (Gee et al., 2013). Additionally, the social brain experiences rapid growth in adolescence (Blakemore, 2012). The social brain is involved in the process of mentalizing which has been linked to medial prefrontal cortex activity showing decreased activity from adolescence to adulthood (Blakemore, 2012), highlighting again the critical role of changing prefrontal cortex activity throughout development. However, this decreasing activity in adolescence is still not well understood and some researchers have proposed that it might be due to a change in the strategy for mentalizing or a result of synaptic pruning (Blakemore, 2012). More studies are needed but the period of adolescence is especially difficult to investigate as the state of sexual maturity has a high impact on developmental trajectories and the onset of puberty varies greatly between individuals (Houston et al., 2014).

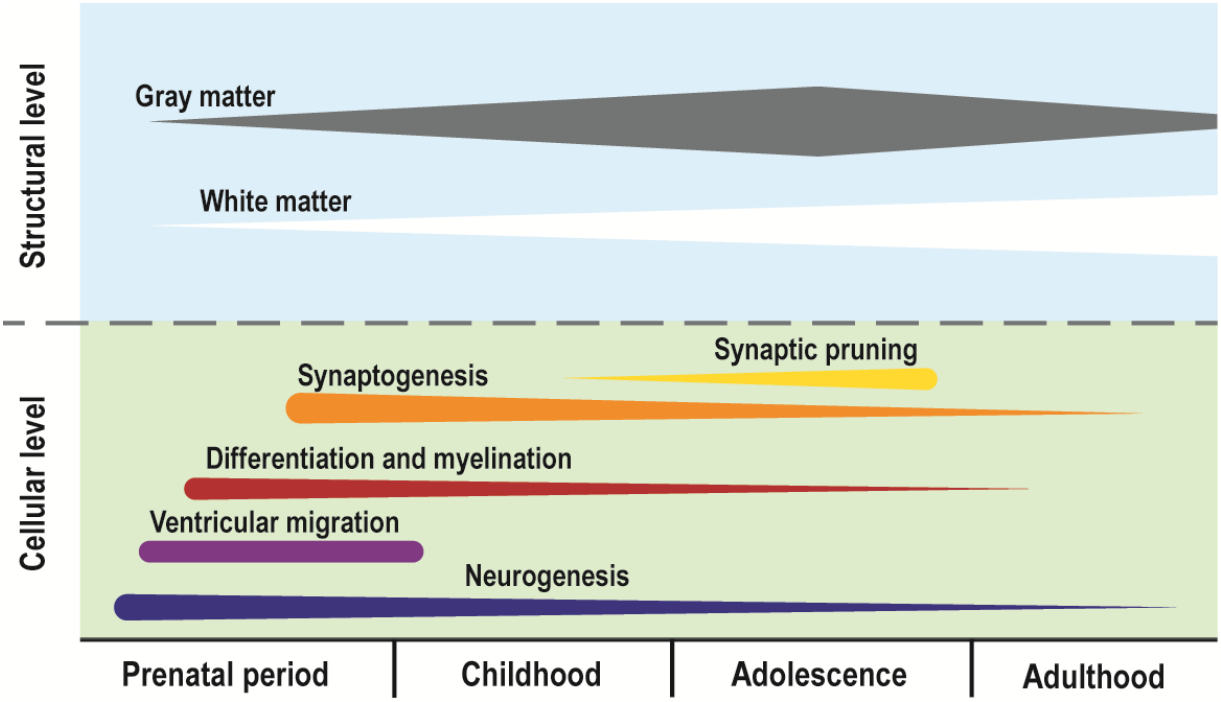


Figure 1: Illustration of brain development throughout life. Schematic illustration of structural and cellular changes throughout life. **Above**: White matter volume increases steadily across life whereas gray matter volume increases in childhood, peaks in adolescence, and decreases afterward. **Below**: Changes during brain development on the cellular level. Neurogenesis starts in the early prenatal period, followed by ventricular migration of neurons, which then differentiate into different cell types enabling myelination of neurons. Cell differentiation is followed by cell maturation and synaptogenesis. In reduced form, neurogenesis persists into adulthood, cell differentiation, myelination, and synaptogenesis persist as well. Adolescence is marked by increased synaptic pruning.

In adulthood, brain development is considered complete as brain structures and connections are fully developed (Lupien et al., 2009). However, small neuronal connections are still being formed or erased (Shors et al., 2012), showing that brain plasticity is a lifelong process. During aging, over time more connections are being erased and thus memories and tasks are being forgotten more often (Lupien et al., 2009). This natural neurodegeneration becomes more prominent in psychiatric disorders like Alzheimer`s or Parkinson`s disease where a rapid loss of neurons leads to severe cognitive, behavioral, and mental problems (Burns & Iliffe, 2009; Sveinbjornsdottir, 2016).

In summary, the human brain undergoes immense changes on the structural and functional level especially during childhood and adolescence that are marked by times of great growth and strengthening of connections critical to building a mature brain. The white matter volume steadily increases throughout development, reflecting the increasing number in connections and circuitries being formed and strengthened (**Figure 1**). However, these structural and functional changes are apparent manifestations of developmental changes on the cellular and molecular

level and, consequently, a deeper insight is needed to fully understand processes of brain development throughout life.

1.1.2. Cellular and molecular level

In the first two trimesters of pregnancy, the prenatal brain is built by different periods of synaptogenesis following an inside-out gradient (Beattie & Hippenmeyer, 2017; Nadarajah et al., 2001; Rakic, 1972), meaning that radial migration of cortical neurons starts from the ventricular zone (origin) to the cortex forming layers (Beattie & Hippenmeyer, 2017). After cell migration, the neuronal progenitor cells differentiate into neurons, astrocytes, and oligodendrocytes (Martínez-Cerdeño & Noctor, 2018). Cell differentiation is followed by cell maturation (e.g. forming dendrites) and synaptogenesis (connecting with other cells) (Kolb & Gibb, 2011) (**Figure 1**). In the third trimester, the two hemispheres synchronize and connections are formed between subcortical and cortical regions (Lagercrantz, 2016). Synaptogenesis and the formation of connections are dependent on sensory input leading to enhanced or reduced synaptogenesis, respectively (Chaudhury et al., 2016; Lagercrantz, 2016). In childhood, the brain experiences high synaptic plasticity marked in the thickening of gray and white matter in cortical and subcortical regions (Houston et al., 2014). Contrastingly, adolescence is a time of synaptic pruning removing connections that are not used regularly (Shors et al., 2012). Disruptions of synaptic pruning can lead to severe impairments in mental processes (Chaudhury et al., 2016; Minshew & Williams, 2007). Interestingly, neurogenesis still occurs in the adult hippocampus and is an important process to maintain a flexible brain (Shors et al., 2012) (**Figure 1**).

Besides neurons, microglia are important cells during brain development as well. Microglia, the macrophages of the brain, express receptors for a wide range of immune molecules and are most active during mid-gestation until the perinatal period. Consequently, microglia play an important part in influencing neuronal numbers (by releasing e.g. growth factors) (Ueno et al., 2013), early wiring of neuronal circuits (Thion et al., 2018) and are even relevant in acquiring certain sex-specific patterns (Lenz et al., 2013). Microglia, therefore, might be involved in shaping sexually dimorphic brain circuitries underpinning their important impact on early brain development (Bilbo & Schwarz, 2012; Thion et al., 2018). Of note, post mortem studies on fetal and infant brain tissue indicated that during early migration of neurons and oligodendrocytes, microglia remove neurons that have falsely migrated into the white matter. An increase in microglial activity in the white matter, therefore, acts protective against injuries (Billiards et al., 2006; Verney et al., 2010).

On the molecular level, glucocorticoids (GCs) are highly important for brain maturation as they initiate terminal maturation, remodel axons and dendrites, and affect cell survival (Kapoor et al., 2006; Lupien et al., 2009). GCs bind to glucocorticoid receptors (GRs expressed by the *NR3C1* gene) which control e.g. the cells' metabolism and immune response (by up-regulation of anti-inflammatory protein expression). Also, GCs can act as transcription or repression factors and are therefore able to regulate gene expression patterns during critical developmental periods (Champagne et al., 2009). Thus, when increased GC levels up-regulate the expression of anti-inflammatory proteins in fetal development, this up-regulation can result in abnormal brain development causative for neurodevelopmental disorders (Champagne et al., 2009). Interestingly, neurotransmitters, hormones, and cytokines are all involved in processes as cell differentiation and migration, forming of synapses, and programmed cell death. Therewith, cytokines are able to enhance or impair hippocampal neurogenesis throughout brain development, respectively (Rolls et al., 2007). Moreover, given their anti-inflammatory or pro-inflammatory roles during an immune response, cytokines can induce physiological changes leading to so-called sickness behavior (e.g. fever, increased sleep, reduced food, and water intake) (Dantzer & Kelley, 2007).

On the DNA level, epigenetic mechanisms can also impact development. The two main mechanisms are histone modifications and DNA methylation which, importantly, allow for heterogeneity between genetically homologous cells by regulating gene expression (Jaenisch & Bird, 2003; Qiu, 2006); further described in **1.2**). Most interestingly, epigenetic changes are reversible throughout life and highly subjective to environmental influences (Jaenisch & Bird, 2003). Given the dynamic properties of epigenetic programming, they are highly important during brain development and maturation. Modifications such as altered DNA methylation are important for certain dynamic neuronal functions as they enable region-specific gene expression and consequently region-specific neuronal growth (Dennis & Levitt, 2005; Shin et al., 2014). As DNA methylation patterns are able to change as a response to environmental factors, they hold great potential for programming effects in early brain development. However, the study of epigenetics in brain development is still new and innovative technologies are still evolving, waiting to identify epigenetic processes involved in normal brain development.

Taken together, the brain development is composed of distinct periods of growth marked by harmonized processes on cellular and molecular levels as well as on structural and functional levels. Periods of excessive brain growth and maturation are most pronounced in childhood and adolescence. However, molecular processes impacting brain growth, are highly susceptible to environmental factors affecting brain development and potentially resulting in impairments.

Epigenetic programming might prove to be the mediator between exposure and alterations. But before drawing this conclusion, the consequences of stress exposure, as one critical environmental factor, on brain development have to be disentangled to identify periods vulnerable to disruptions resulting in neuronal implications.

1.1.3. Brain development and stress

The brain undergoes certain periods of growth marked by an interplay of different processes. As these processes are susceptible to external influences, periods of growth are vulnerable to disruptions leading to impairments. The experience of stress during sensitive periods of brain growth especially increases the risk for long-lasting impairments (Andersen & Teicher, 2008; Lupien et al., 2009). Induced implications become apparent in functional and structural alterations first. Given the prolonged maturation of the hippocampus, amygdala, and prefrontal cortex, these regions are thus most vulnerable to demonstrate permanent alterations after stress exposure (Lupien et al., 2009) (**Figure 2**). Consequently, early life stress (ELS) induces neuroanatomical changes such as reduced volumes of the hippocampus, amygdala, and the prefrontal cortex leading to deficits in memory, emotional perception, and behavior (Hart & Rubia, 2012). As a result of reduced hippocampal volume and decreased synaptic plasticity, ELS leads to an increased amygdala: hippocampus ratio associated with pediatric depression and anxiety (Andersen & Teicher, 2008).

As mentioned above, the amygdala-prefrontal cortex circuitry is especially vulnerable during adolescence with the amygdala showing high reactivity to emotional stimuli before the adult-like pattern of connectivity is formed (Tottenham & Galván, 2016). This increased reactivity indicates a time of instability and thus vulnerability. For example, adverse caregiving as a form of early psychological stress or physical abuse results in hyper-reactivity of the amygdala towards emotional stimuli during adolescence (Tottenham & Galván, 2016). Amygdala hyper-reactivity is associated with depression and posttraumatic stress disorder (PTSD) (Tottenham & Galván, 2016) whereas weaker amygdala-prefrontal cortex connectivity is associated with symptoms of anxiety (Gee et al., 2013; Tottenham & Galván, 2016). On the cellular level, ELS can induce alterations in oligodendrocytes within the white matter of the prefrontal cortex possiblity resulting in severe cognitive impairments (Tanti et al., 2018). Moreover, as adolescence is a time of synaptic pruning, disruptions in synaptic pruning processes can lead to severe impairments in mental processes associated with psychopathologies such as schizophrenia (Chaudhury et al., 2016; Minshew & Williams, 2007).

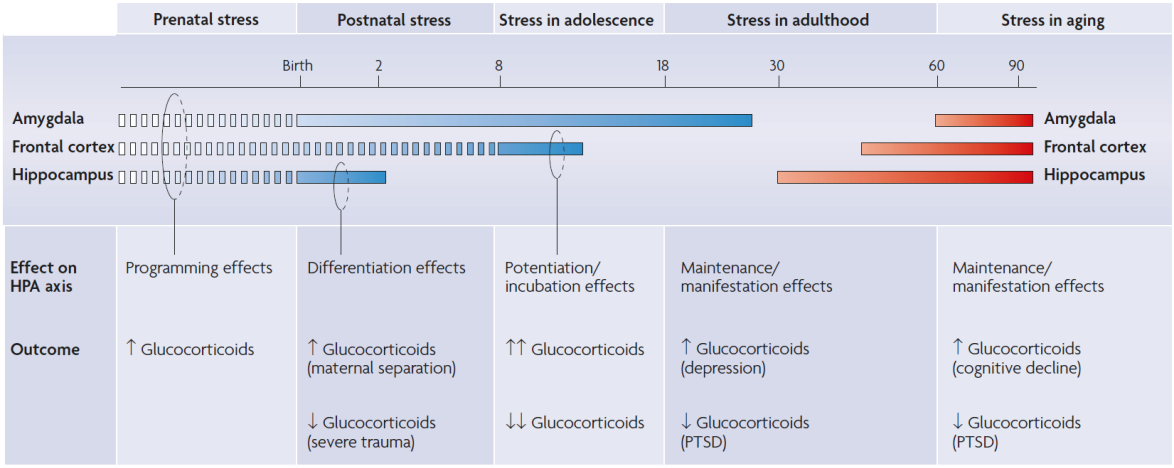


Figure 2: Consequences of stress exposure during sensitive periods of brain development. Effects of stress exposure during different sensitive periods of brain development. Depending on brain region developing (blue) or declining (red) different consequences on hypothalamus-pituitary-adrenal (HPA) axis function and altered glucocorticoid (GC) levels emerge leading to severe impairments e.g., post-traumatic stress disorder (PTSD). Broken bars represent periods when regions are most sensitive to GC exposure, solid bars represent periods of accelerated growth. Amygdala, frontal cortex and hippocampus experience prolonged development and are thus most sensitive to stress exposure. Adapted from Lupien *et al.* (2009).

On the molecular level, stress-related hormones (e.g. GCs) play a key role in sculpting the brain as they play an important part in normal brain maturation (Andersen & Teicher, 2008; Kapoor et al., 2006). However, altered levels of GCs during critical periods of growth impact brain development leading to retarded growth of specific regions such as the hippocampus (Champagne et al., 2009), especially as the hippocampus, amygdala, and prefrontal cortex show the greatest GC receptor density (Dziedzic et al., 2014; Sapolsky et al., 2000; Tyborowska et al., 2018) (**Figure 2**). Elevated levels of GCs, consequently to stress exposure, then affect the hypothalamic-pituitary–adrenal (HPA) axis (Frodl & O'Keane, 2013).

As the main system regulating the stress response, the HPA axis is highly relevant in determining the effects of stress exposure on brain development (Aisa et al., 2008; Heim et al., 2008; Lupien et al., 2009). In brief, neurons of the hypothalamus release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP). This triggers the pituitary gland to release adrenocorticotrophic hormone (ACTH) which then induces the adrenal cortex to produce GCs (Lupien et al., 2009). ACTH and CRH release is regulated by GCs as they bind to the GR and the mineralocorticoid receptor (MR). Therewith, GCs determine the responsiveness of the HPA axis to stress (Lupien et al., 2009). Animal studies using maternal separation revealed that ELS resulted in elevated GC plasma levels, increased CRH binding sites in the prefrontal cortex, hippocampus, hypothalamus, amygdala, and cerebellum together with increased HPA axis activity and anxiety as well as increased CRH mediated hippocampal loss of spines (Anisman et al., 1998; Lupien et al., 2009). In children, elevated levels of GCs could be observed after

daily separation from their parents e.g. daycare (Lupien et al., 2009). Interestingly, early severe deprivation, neglect, or abuse resulted in lower basal GC levels in children potentially as a consequence of a downregulation of the HPA axis in response to chronically elevated levels of CRH (Lupien et al., 2009). Furthermore, it has been proposed that early GC conditions (e.g. level of stress exposure) set the life-long stress susceptibility via developmental epigenetic programming (further discussed in 1.4.1). This programming might then lead to enhanced vulnerability to psychopathology if early and later life experiences fail to match (Champagne et al., 2009). Therefore, the exposure of the fetal brain to elevated GC levels modifies the HPA axis function and stress reaction for a lifetime (Kapoor et al., 2006). In addition, the HPA axis shows a prolonged activation in response to a stressor in adolescents compared to adults resulting in prolonged exposure of the brain to GCs during a critical time of maturation (McCormick & Mathews, 2007). This might render adolescents particularly vulnerable to severe consequences of stress exposure and seems to induce permanent changes in cognition and addictive behavior (Galván, 2017).

To summarize, severe stress exposure during sensitive periods of brain development increases GC levels impacting HPA axis function and therewith induce developmental programming to stress susceptibility. Altered HPA axis function further affects neuronal growth resulting in structural and functional impairments. A dysfunction in the HPA axis, as well as functional impairments in important neuronal networks, are furthermore linked to several disorders such as depression and anxiety (Pariante & Lightman, 2008; Roberts et al., 2015) highlighting psychiatric disorders as disorders of the brain. Interestingly, impairments in disorders seem to align with neuronal processes most important during the time of symptom manifestation.

1.2. Psychiatric disorders

Psychiatric disorders can be defined as disturbances in personal cognition, emotion regulation, or in behavior controlled by mental processes that are clinically noticeable and lead to deficits in everyday life (American Psychiatric Association, 2013). These disturbances manifest as exaggerated or reduced normal behavior. For example, in depression, negative affect is exaggerated whereas the feeling of euphoria is excessive in manic episodes of bipolar disorders. In anxiety, normal fear becomes overwhelming and cognitive skills might be reduced in schizophrenia as well as social skills are reduced in autism spectrum disorder. Even though most psychiatric disorders show high heritability, disentangling their genetic basis has proven to be difficult (Burmeister et al., 2008; Hoehe & Morris-Rosendahl, 2018). One challenge in identifying the genetic basis of disorders is that certain genetic risk factors interact with

environmental factors, especially toxic stress, and this interaction is then causative for psychopathology (Burmeister et al., 2008).

1.2.1. General information on different disorders

Psychiatric disorders are classified in different categories based upon their main symptoms and impairments but, amongst others, due to the typical age of onset (American Psychiatric Association, 2013) proposing that certain periods in life are more vulnerable to certain impairments. In terms of brain development, impairments in disorders seem to align with neuronal processes being most important during that time, e.g., in infancy, fundamental networks are being formed while during adolescence decision making, reward processing and, the social brain experience enhanced maturation. On the diagnostic side, neurodevelopmental disorders such as autism spectrum disorder, attention deficit hyperactivity disorder (ADHD), and tic disorders begin in infancy whereas personality disorders like borderline disorder, psychotic disorders as schizophrenia as well as substance-related and addictive disorders mostly manifest during adolescence and early adulthood (American Psychiatric Association, 2013). Neurocognitive disorders like delirium and dementia are most prominent in older individuums and are marked by a progressive loss of neurons (American Psychiatric Association, 2013). For stress-related disorders like anxiety disorders, posttraumatic stress disorder (PTSD), and depression, the age of onset varies. Mostly, anxiety disorders manifest already in childhood, whereas the age of onset of PTSD and depression is predominantly in adolescence and early adulthood (American Psychiatric Association, 2013). However, later disorders can manifest during every stage of development. For example, depression also manifests in older individuals after the loss of their partner or in women after childbirth. PTSD can develop in adolescents after traumatic experiences but is also frequently diagnosed in war veterans (Andreasen, 2010). However, anxiety disorders and depression are especially interesting in the context of brain development as both disorders have a high global prevalence, show a high transition between diagnosis and, depending on the (sub)type of disorder, have multiple times of onset throughout life (Costello et al., 2003; Global Burden of Disease Study 2013 Collaborators, 2015; Kessler et al., 2005; Nieto et al., 2016; Zimmerman et al., 2000).

Given the high prevalence of affected, the etiology and neuronal alterations of psychiatric disorders have been repeatedly studied indicating a gene x environment interaction leading to neuronal implications with reduced hippocampal and prefrontal cortex volumes and a dysregulation of the HPA axis predominantly being reported (Beach et al., 2016; Belleau et al., 2019; Malykhin et al., 2010; Maron & Nutt, 2017; Oakes et al., 2017; Ota et al., 2018; Palazidou, 2012; Roberts et al., 2015). On the molecular level, genetic risk variants have been

revealed by genome-wide association studies (GWAS) (Aragam et al., 2011; Sullivan et al., 2009) as well as epigenetic modifications in DNA packaging (Farrell & O'Keane, 2016) reinforcing an interplay between genetic risk variants and environmental factors via epigenetic modifications.

To sum up, psychiatric disorders are a global burden with a high lifetime prevalence for the affected. Categorizing disorders based on their typical age of onset highlights the importance of vulnerable windows in brain development for determining psychopathology. Even though disorders are characterized based on distinct symptoms and resulting impairments, they share common risk factors and environmental influences. One common environmental risk factor for the development of psychiatric disorders is severe stress exposure. Severe exposure might interact with certain genetic risk factors leading to psychopathology (Burmeister et al., 2008).

1.2.2. Psychiatric disorders and stress

Severe stress exposure is a known risk factor to increase the development of most psychiatric disorders, especially of MDD, bipolar disorder, PTSD, and schizophrenia (Matosin et al., 2017; Vinkers et al., 2015). In terms of ELS, different subtypes of ELS even increase the risk for certain psychopathologies. More precisely, physical and sexual abuse, as well as unspecified neglect show the highest risk for developing mood and anxiety disorders, whereas emotional abuse is associated greatest with the development of personality disorders and schizophrenia. The development of a personality disorder is furthermore associated with physical neglect (Carr et al., 2013). Interestingly, four out of five subtypes were associated with an increased risk to develop an anxiety disorder (Carr et al., 2013). However, stress can be harmful not only when experienced early in life but also throughout life. Moreover, pathological consequences can arise from one very traumatic event (e.g. loss of a caregiver or a natural disaster) or from chronic stress exposure (e.g. repeated bullying, domestic violence, or social isolation) (American Psychiatric Association, 2013). The time of exposure is also critical in determining the resulting psychopathology e.g., sexual abuse before the age of 12 increases the risk to develop depression whereas sexual abuse after the age of 12 increases the relative risk to develop PTSD (Schoedl et al., 2010).

However, independent of disorders, early childhood is the most critical time concerning stress exposure as early exposure changes the sensitivity towards the pathogenic effects of stress throughout life (Kendler et al., 2004). This hypothesis is reflected in the diathesis-stress model of depression, also known as the two-hits model, stating that early adverse life events (first hit) make the brain vulnerable whereas a stressor later (second hit) is triggering psychopathology

(Mc Elroy & Hevey, 2014; Worlein, 2014). This hypothesis is in line with the consequences of cumulative stress leading to more severe symptoms (Geronazzo-Alman et al., 2017; Suliman et al., 2009). Moreover, it is hypothesized, that every new stress exposure or psychiatric episode activates neurotoxic pathways resulting in a decline of brain structures along with the progress of the disorder (Belleau et al., 2019).

Thus, stress or traumatic events experienced over a prolonged time or during sensitive periods increase the risk to develop psychiatric disorders. Depending on the time of exposure, different symptoms develop. However, in humans, exposure and resulting neurological consequences are difficult to disentangle as different environmental factors influence brain development. Furthermore, neurobiological alterations in humans can solely be analyzed on structural and functional levels (e.g., imaging techniques) or in postmortem tissue. Therefore, animal models enable us to overcome this obstacle as they allow for a controlled environment, to determine the type and time of exposure and the study of induced neurobiological changes on the molecular level causative for psychopathology.

1.3. Early life stress in animals

Animal models of ELS allow investigating longlasting neurobiological impairments on the molecular level. However, concerning developmental studies, some things have to be considered when studying animals, especially when findings should be translatable to humans. Developmental studies frequently are carried out in rodents as the developmental stages are defined in a similar matter to humans and their shorter life cycle and high reproduction rate enable a fast generation of results throughout different ages as well as transgenerational studies (Ellenbroek & Youn, 2016). However, rodents are born prematurely, and thus the brain maturation during the first postnatal week is comparable to the human prenatal brain maturation in the third trimester (Kapoor et al., 2006). The most frequent paradigm to induce ELS in animals is maternal separation (MS) since it results in behavioral and neurobiological impairments similar to those seen in humans after ELS such as increased anxiety or depression (Carr et al., 2013; Leussis et al., 2012), increased GC levels (Frodl & O'Keane, 2013; Plotsky & Meaney, 1993) and reduced neuronal plasticity (Lupien et al., 2009; Teicher et al., 2003).

1.3.1 Maternal separation: Effects on pups

In the paradigm of MS, the pups are being separated from the dam for several hours a day during the early postnatal days (Lehmann & Feldon, 2000). For MS, the days chosen for

separation settle the implications on behavior and neurobiology (Freund et al., 2013; Peña et al., 2017). As there generally is a high diversity concerning the protocols to induce MS, each varying in postnatal days chosen for separation, in hours daily, and the total duration of separation, inconsistent results have been reported in mice (Tractenberg et al., 2016) and rats (Lehmann & Feldon, 2000). For a more coherent overview of induced alterations of MS exposure, this section will, therefore, focus on results from studies in rats using a certain paradigm as this paradigm was the one used in the studies included in this thesis. In brief, pups are separated from the dam and littermates for 4 hours every day from postnatal day 2-20. Interestingly, MS reveals behavioral and neuronal implications reported in the preceding sections on stress effects on brain development and psychiatric disorders.

In terms of synaptic plasticity, the consequences of MS throughout development show reversed effects over time underlining not only the crucial time point of exposure but also the time of examination. Thus, MS might impact neuronal plasticity, respectively. While synaptophysin-positive cells (as a marker of plasticity) within the hippocampus does not increase as a consequence of MS at postnatal days 25 and 40, significantly less synaptophysin was found by postnatal days 60 until 100 when compared to controls (Andersen & Teicher, 2004). Another marker of neural plasticity is perineuronal nets (PNN). These are extracellular matrix structures that e.g. influence synaptic plasticity, protect neurons from damage, and stabilize synapses (Reichelt et al., 2019). Damage to PNN renders the neurons vulnerable and leads to severe deficits in e.g. cognition (Reichelt et al., 2019). Therefore, the study of PNN after MS can reveal helpful insights into the effects of ELS on synaptic stability. A recent study investigated PNN and GABAergic neurons in the prefrontal cortex and the basolateral amygdala after MS in juvenile, adolescent, and adult rats revealing that MS induces sex- and age-specific alterations (Gildawie et al., 2020). More precisely, MS delays PNN formation in juvenility but the delay does not persist until adolescence. In adolescents and adults, the intensity of PNN shows age and region-specific alterations between males and females indicating an important role of PNN formation surrounding GABAerig neurons for implications on neuronal plasticity after MS (Gildawie et al., 2020). Moreover, MS induces sex-specific alterations in amygdala-prefrontal cortex connectivity resulting in strengthened trajectories in juvenility and adolescence (Honeycutt et al., 2020). The trajectories of females exposed to MS were already affected in juvenility whereas alterations in males were obvious at first in adolescence. In both sexes, MS leads to earlier maturation of the amygdala-prefrontal cortex connectivity. The accelerated amygdala-prefrontal cortex connectivity also correlates with increased anxiety (Honeycutt et al., 2020). Concerning the implications of MS on prefrontal cortex function, consequences of

MS on addiction-related neural networks have been analyzed. Therefore, the expression of the dopamine (D)1 and D2 receptors in the prefrontal cortex was investigated in juvenile, adolescent and adult rats after MS (Brenhouse et al., 2013). In controls, D1 and D2 receptor expression in prefrontal cortex neurons peaks in adolescence but in MS, this peak is missing. The missing peak of prefrontal cortex D1 and D2 receptor expression during adolescence might be causative of increased reward-seeking behavior than usual for this period and thus increase vulnerability for addiction-related behavior after early exposure (Brenhouse et al., 2013).

Observing the consequences of MS on adolescents and adults reveals sex-specific sensitive periods when examining depressive-like behavior as well (Leussis et al., 2012). Exposing MS rats to either inescapable, escapable electroshock, or no shock in the triadic model of learned helplessness, therewith learned helplessness results in sex-dependent changes with female adolescents experiencing rather motivational impairment during the no shock condition and males showing a loss of controllability in the escapable shock condition. Adult females do not show helpless behavior anymore. Depressive-like behavior was, moreover, associated with a reduction in prefrontal cortex GABAergic neurons (Leussis et al., 2012).

However, MS is considered a pre-weaning stressor since the pups are still cared for by their mother, and thus, only reflects the consequences of ELS during early childhood. A frequently used post-weaning stressor allowing to examine consequences of exposure during late childhood to early adolescence is social isolation (ISO) where the animal is single-housed over a defined period. Consequently, ISO after weaning for a total of 13 weeks leads to increased anxiety and alteration of the HPA axis with increased corticosterone release in males but not in females compared to group-housed controls (Weiss et al., 2004). Interestingly, even when three weeks of ISO are followed by two weeks of group-housing, adults still show increased anxiety and fear as well as reduced social interaction compared to controls (Lukkes et al., 2009). This indicates that the consequences of ISO are severe and long-lasting as well.

Taken together, consequences induced by MS are well studies on behavioral and neurobiological levels. Altered synaptic plasticity within and between specific regions as well as changed amygdala-prefrontal cortex circuitry activity and reduced activity towards rewarding stimuli have been reported in humans experiencing ELS as well. Results from MS studies can thus deliver deeper insights into the molecular changes induced by early exposure by e.g., studying altered receptor expression, synaptic plasticity, and functionally different neurons which ultimately are responsible for the found implications. Exposure after weaning by ISO induces long-lasting alterations and thus, ISO proves to be a reasonable stressor to

examine the effects of post-weaning stress. Further, the effects of exposure are changing throughout life reinforcing the need to investigate different developmental stages after exposure. However, during MS, the pups are being separated from their mother and since the early postnatal time is a sensitive time not only for pups but also for dams, repeated separation also affects dams. Moreover, maternal health is important for offspring development as early maternal behavior impacts the offspring's stress response (Weaver et al., 2004).

1.3.2. Maternal separation: Effects on dams

For pups, the separation occurs during the sensitive early postnatal days while for dams, the separation takes place during the sensitive postpartum time (Brummelte & Galea, 2010, 2016). So far, only a few studies are focussing on maternal consequences of MS and again, as different MS paradigms are used, the results are frequently inconsistent. However, the overall results indicate severe consequences of MS on dams as well (Alves et al., 2019; Boccia et al., 2007; Orso et al., 2019; Sung et al., 2010). Several studies found that MS increases anxiety depressive-like behavior in dams measured (Bousalham et al., 2013; Maniam & Morris, 2010; Sung et al., 2010). Studies also reveal changes in the reward system after MS as dams tend to greater drug-abuse behavior in a cocaine self-administration task (Moffett et al., 2006). Besides behavior, neurobiological impairments have been observed as well. MS leads to an increased expression of *Crh* mRNA (Maniam & Morris, 2010) as well as a decrease of cell proliferation along with increased hippocampal cell apoptosis (Sung et al., 2010). Furthermore, MS might even induce similar pathologies in dams as seen in postpartum depression e.g. increased anxiety and depression, altered maternal care as well as similar neurobiological changes. However, for a broader analysis of the consequences of MS on dams, studies should include both, behavioral and neurobiological parameters. Therewith, studies could determine a possibly induced psychopathology in dams. Consequently, more studies are needed to investigate the maternal side of MS before concluding the resulting psychopathology.

Behavioral and neurobiological alterations in dams after MS are important to investigate as they highlight the postpartum period as another vulnerable time. Impairments in neuronal plasticity and the stress system implicate severe alterations after exposure. Moreover, these changes are important to consider when investigating consequences on offspring as maternal health reflected in maternal care has a significant impact on offspring development. The level of maternal care towards their pups during the early postnatal days determines the offspring's stress reaction via epigenetic programming for life (Orso et al., 2018; Weaver et al., 2004). Thus, maternal behavior also proves to be an environmental factor influencing gene expression via epigenetic modifications.

1.4. Epigenetics

Generally, differences in gene expression (through epigenetic mechanisms) allow heterogeneity between genetically homologous cells (Jaenisch & Bird, 2003). Thus, epigenetic modifications are mechanisms that allow changes in phenotype without changing the genotype. They are alterations in the accessibility of genomic DNA that result in altered gene function and expression (Razin & Cedar, 1991; Razin & Riggs, 1980). The two main forms of epigenetic modifications are histone modification and DNA methylation (Qiu, 2006) (**Figure 3**). So far, the most frequently studied epigenetic mechanism is DNA methylation which is a post-replication modification of the DNA sequence by the addition or removal of a methyl group at a CpG site (Jaenisch & Bird, 2003) (**Figure 3**). The degree of gene methylation then usually correlates negatively with the degree of gene activity e.g. a high degree of gene methylation is associated with less gene expression (Razin & Riggs, 1980).

Epigenetic modifications can lead to harmful silencing or overexpression of certain genes causative for certain phenotypes such as cardiovascular and neurodegenerative disorders, cancer, and also psychiatric disorders (Mahgoub & Monteggia, 2013; Santos-Rebouças & Pimentel, 2007). Further research investigating epigenetic alterations can thus provide important insights into the pathologies of diseases. So far, the most prominent clinical research field of epigenetic modifications is in cancer epigenetics, where possibilities of epigenetic cancer therapy are already bein investigated (Sharma et al., 2010). However, the field of psychiatric epigenetics is developing rapidly as well. One of the most studied environmental factors influencing both, DNA methylation and psychopathology, is stress.

1.4.1. DNA methylation and stress

In general, DNA methylation can be influenced by multiple environmental factors such as physical activity (Alegría-Torres et al., 2011), prenatal diet (Rijlaarsdam et al., 2017), smoking and alcohol consumption (Brückmann et al., 2016; Philibert et al., 2012) as well as stress exposure (Mundorf & Freund, 2019).

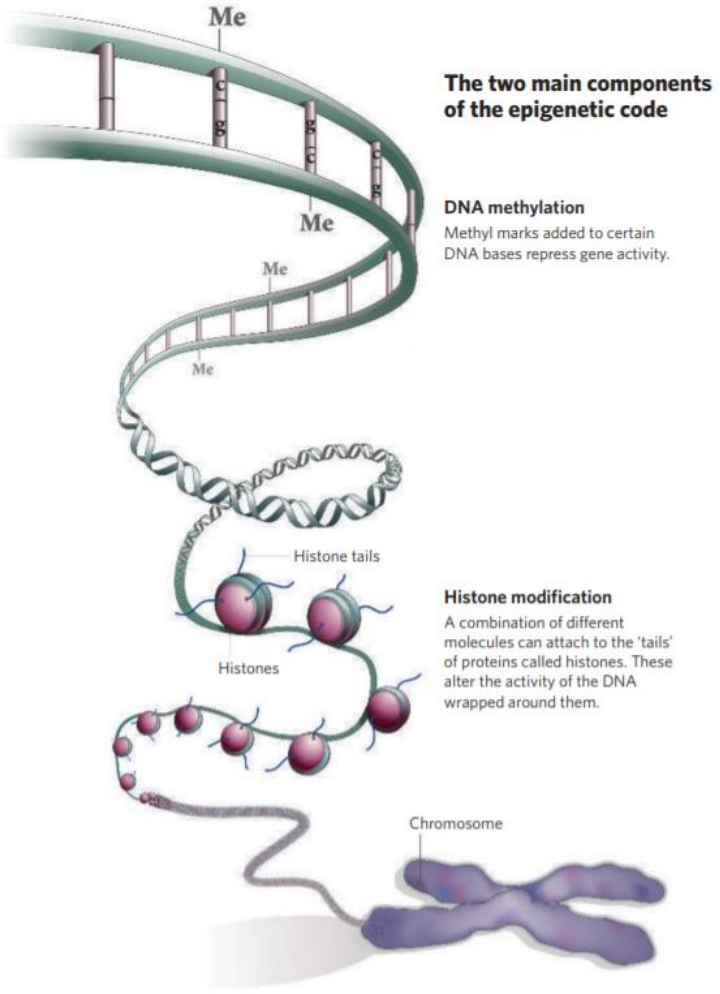


Figure 3: Epigenetic modifications. The two main epigenetic modifications: DNA methylation and histone modifications.

The implication on the individual's mental health depends on the time of exposure e.g. whether the exposure occurred prenatally, in early childhood, or adolescence (Matosin et al., 2017). The serotonin transporter gene (*SLC6A4*), the *NR3C1* gene, and the *FKBP5* gene (involved in the immune system and interacts with *NR3C1*) prove to be main candidates for stress-induced alterations in gene methylation patterns (Vinkers et al., 2015). Genes like the *oxytocin receptor gene* (*OTXR), BDNF*, and *NR3C1* have been repeatedly associated with prenatal stress or low maternal care in human and rodent studies (Beery et al., 2016; Behnia et al., 2015; Cecil et al., 2014; Roth et al., 2009; Weaver et al., 2004). For example, higher prenatal risk exposure has been linked to increased *OXTR* methylation at birth remaining stable until the age of 7 years and to higher callous-unemotional trails in adolescence (Cecil et al., 2014). Thus, *OXTR* methylation seems to mediate callous-unemotional trails, a risk factor for early-onset conduct problems (Cecil et al., 2014). Additionally, preterm birth, as perinatal stress, results in significantly higher *OTXR* promotor methylation in fetal membrane tissue in preterm birth

children and increases the risk for autism spectrum disorder (Behnia et al., 2015). Prenatal stress, induced by restraint stress in pregnant rats, leads to decreased *Crh* promotor methylation, hyper-responsiveness of the HPA axis, and increased anxiety in adolescent offsprings indicating that prenatal stress alters normal HPA axis functioning via DNA methylation (Xu et al., 2014).

To investigate the link between exposure, methylation, and psychopathologies, frequent early life exposure is correlated with DNA methylation patterns in adolescence or adulthood. In a small sample study including 46 non-diagnosed young adults, researchers investigated the link between ELS, psychopathology, and *NR3C1* gene methylation in blood cells (Radtke et al., 2015). Childhood maltreatment and psychopathologies were assessed by questionnaire-based interviews. Maltreatment was associated with both, increased *NR3C1* gene methylation and pathopsychological symptoms of borderline personality disorder. Interestingly, maltreatment and *NR3C1* methylation showed an additive effect on borderline personality disorder symptom severity (Radtke et al., 2015). Another study investigated general DNA methylation patterns in adolescence after childhood stress exposure revealing altered methylation patterns after stress in infancy and preschool (Essex et al., 2013). Of note, as the data used in this study is part of an ongoing longitudinal study, the stress assessment was not conducted retrogradely but prospectively and thus, is more precise (Barker et al., 2017). Interestingly, maternal and paternal stressors had different sensitive windows in terms of methylation changes, with maternal stressors in infancy and paternal stressors in preschool years being predictive for DNA methylation changes (Essex et al., 2013).

As mentioned in **1.3.,** ELS in rodents is frequently induced by MS. When studying DNA methylation changes after ELS, MS is therefore used to assess changes in the rodent brain. Interestingly, MS studies identified similar changes in gene methylation as human studies. For example, investigating the consequences of MS on behavior and gene methylation in mice showed increased anxiety in adulthood along with altered gene methylation patterns in the hippocampus as increased *Nr3c1* and *Avp* methylation after MS (Kember et al., 2012). MS furthermore leads to decreased methylation of *Avp* (Murgatroyd et al., 2009) and decreased methylation of *Crh* in the hypothalamus (Chen et al., 2012).

To summarize, multiple studies in animals and humans have reinforced the important role of DNA methylation modifications in regulating the consequences of stress exposure. Studies found altered methylation patterns of genes important for brain maturation, plasticity, and HPA axis regulation after exposure causative for later psychopathologies. Animal studies can

advance the field of epigenetic modifications and their neurobiological implications. However, independent of exposure, altered DNA methylation is reported in different psychiatric disorders indicating a potential use of methylation as a biomarker of psychopathology or treatment success.

1.4.2. DNA methylation and psychiatric disorders

Epigenetic modifications such as DNA methylation might be the mediating link between stress exposure and psychiatric disorders (Barker et al., 2017; Klengel et al., 2014; Matosin et al., 2017; Vinkers et al., 2015). Stress exposure modifies the methylation patterns of certain genes resulting in harmful gene silencing or overexpression causative for psychopathology. However, altered DNA methylation is not always disorder-specific as some genes have been linked to multiple psychiatric disorders. The *SLC6A4* gene, for example, was found to be differently methylated in patients with bipolar disorder, MDD, PTSD, schizophrenia, ADHD, and obesity (Palma-Gudiel & Fañanás, 2017). Likewise, altered methylation of *NR3C1* and *FKBP5* has been linked to MDD, PTSD, and AD (Argentieri et al., 2017; Roberts et al., 2019). Moreover, increased *NR3C1* methylation was found in patients with borderline personality disorder, and increased methylation of *FKBP5* was associated with bipolar disorder patients (Argentieri et al., 2017) underlining a fundamental role of these genes in mental functioning.

Translational findings further strengthen the impact of modified DNA methylation being causative for psychopathology. Borderline personality disorder patients, with more than half of the included patients having experienced childhood trauma, showed an increased *BDNF* promotor methylation (Thomas et al., 2018). Similarly, increased *BDNF* methylation in the prefrontal cortex of adult rats after abusive maternal care was reported (Roth et al., 2009). Interestingly, the treatment of these animals with DNA methylation inhibitors led to decreased *Bdnf* methylation and normalized *Bdnf* mRNA expression (Roth et al., 2009) aligned with findings that psychotherapy also significantly decreased *BDNF* methylation in patients (Thomas et al., 2018) the study indicates that psychotherapy normalized *BDNF* mRNA expression in patients via inhibiting DNA methylation. In AD, psychotherapy is also reported to reverse the modified methylation pattern of the *SLC6A4* and the *FKBP5* gene in patients with stronger changes in methylation patterns associated with greater treatment response (Roberts et al., 2014, 2019). Besides psychotherapy, pharmacological treatment modifies DNA methylation patterns as well (Boks et al., 2012; Ovenden et al., 2018) giving rise to new treatment targets.

Taken together, studying DNA methylation patterns after exposure and in psychopathology holds great potential for the identification of biomarkers that would not only allow for prediction of treatment outcomes but also enable early detection. Given that certain genes are affected after ELS and in psychiatric disorders in animals and humans strengthening the critical mediating role of DNA methylation. Moreover, animal studies revealed that exposure leads to altered gene methylation causative for neurobiological impairments. The field of psychiatric epigenetics still emerging, rendering innovative new approaches in studying epigenetic influences during development and their neurobiological consequences leading to psychopathology.

1.5. Aim of the thesis

Epigenetic regulation is important in the processes of brain development as processes such as DNA methylation allow for region-specific gene expression and thus, for region-specific neuronal growth. However, stress exposure alters DNA methylation of certain genes that can lead to harmful gene silencing or overexpression during sensitive periods of neuronal growth that, in turn, may result in psychopathological symptoms and impairments. However, even though certain modifications have been revealed, other relevant processes in the context of exposure and psychopathology still have to be examined in detail. To disentangle epigenetic modifications in the context of brain development and psychopathology, the aims of this thesis are the following:

1. Translational investigation of white matter alterations on the structural and molecular level in the context of early life adversity and depression (**Chapter 2**).

2. Identifying sensitive periods for and long-term consequences of early stress exposure on behavior, DNA methylation patterns and neurobiology using human studies and rat models of early life stress (**Chapter 3**).

3. Examining other vulnerable periods of brain development marked by hormonal changes. Therefore, neurobiological, and behavioral consequences after stress exposure during the postpartum time found in mothers after childbirth and in rat dams are investigated (**Chapter 4)**.

4. Unraveling the potential application and validity of DNA methylation and neuronal correlates in psychiatric disorders in studies involving healthy participants and genetically modified mouse models (**Chapter 5**).

To resolve the complex mechanisms involved, in this thesis, different concepts are combined enabling a stepwise approach. Therefore, the thesis contains three comprehensive literature reviews and nine research articles aiming to disentangle epigenetic modifications by identifying sensitive periods, neuronal correlates as well as DNA methylation and gene expression patterns in the context of brain development, psychopathology, and exposure.

Chapter 2 |
White matter alterations in depression

Unraveling the mystery of white matter alterations in depression: A comprehensive study of recent advances

Mate Abraham [1],Annakarina Mundorf *[1] and Nadja Freund [1]

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

*Corresponding author

Review article / Manuscript submitted

Abstract Numerous cortical and subcortical structures have been studied extensively concerning alterations of their integrity as well as their neurotransmitters in depression. However, connections between these structures have received considerably less attention. This review presents results from recent neuroimaging as well as neuropathologic studies conducted on humans and other mammals, providing strong evidence for impaired white matter integrity in individuals expressing a depressive phenotype. This is especially apparent in frontal gyri, as well as in structures establishing interhemispheric connectivity between frontal regions. Translational neuropathological findings point to alterations in oligodendrocyte density and morphology. An important role of early life adversities in the development of depressive symptoms and white matter alterations across species is thereby revealed. We present data indicating that stress can interfere with physiological myelination patterns. Altered myelination is most notably present in regions that are subject to maturation during the stage of development in which exposure to adversities takes place. Moreover, indications of alterations in methylation as well as expression of genes related to myelin synthesis are presented.

Keywords MRI, fractional anisotropy, postmortem, suicide, rodents, MDD, primates, interhemispheric, intrahemispheric

1. Introduction

Major Depressive Disorder (MDD) is a chronic health condition causing considerable distress to affected patients. Moreover, MDD is one of the leading causes of disability worldwide: according to the Lancet Global Burden of Disease from 2017, depressive disorders (comprising MDD and Dysthymia) were the third-largest contributor to Years Lost to Disability (YLDs) on a global scale, preceded only by low back pain and headache disorders(James et al. 2018). Furthermore, MDD is often associated with other diseases, such as anxiety disorders, type II diabetes, chronic back pain, and rheumatic diseases, further highlighting its clinical relevance. (Baerwald et al. 2019; Fava et al. 2000; Eaton et al. 1996; Currie und Wang 2005)

Despite MDD being a large health and socioeconomic burden, to date, there is no universal consensus about its pathogenesis. Numerous studies, both in humans and animals, have been conducted in order to discover the mechanisms underlying the condition (Kathol et al. 1989). Even though there has been a lot of progress in this field, the exact cytological correlates of mood disorders are still not clear (Edgar und Sibille 2012).

For a long time, it was difficult to determine functional and structural changes in the brain of patients suffering from mental disorders as these changes are frequently not clearly seen in post-mortem tissue, and in vivo methods were not capable of identifying alterations. The inventions of new technologies allowing structural and functional investigating of the brain in living individuals revealed significant brain alterations in psychiatric patients. One of the most important instruments that has recently gained increasing relevance to investigate the structures of the brain is magnetic resonance imaging (MRI). MRI provides a way to create high-resolution images of patients´ central nervous system (CNS), thus delivering useful information about imaging biomarkers of depression. To date, this method was mostly used to investigate volumetric aberrations of brain structures in depressed patients. One of the most consistent results in this context is a reduced hippocampus size, as reviewed amongst others by Videbech et al. (Videbech und Ravnkilde 2004). Another aspect that has recently been heavily investigated using new imaging technologies is the alteration of white matter (WM). The advancement of diffusion metrics in MRI has allowed for a more sophisticated investigation of WM architecture, in humans as well as in rodents. The most common method to analyze alterations in diffusion metrics in MDD has been fractional anisotropy (FA), an important tool to model nerve fiber structures.

Preclinical studies conducted on rats and mice, and clinical studies conducted on humans both play an important role in helping us to gain a better understanding of the pathogenesis of MDD. However, only a few reviews have focused on translating findings concerning WM in animal

studies to humans and vice versa (Edgar und Sibille 2012; McNamara und Lotrich 2012). Recent advances in MRI allow us to take a closer look at alterations in diffusion metrics in WM in both clinical and preclinical trials. They furthermore provide the valuable possibility of comparing findings in different species allowing to gain further insight into the reasons underlying these alterations.

In this systematic review, we aim to provide a complete overview of recent advances concerning WM alterations in humans, primates, and rodents. Our review comprises four types of studies: MRI studies conducted on rodents and primates with an animal model of depression, post-mortem studies conducted on rodents and primates with an animal model of depression, human MRI studies in patients with the clinical diagnosis of MDD as well as post-mortem studies conducted on deceased MDD patients. Taking these different types of studies into consideration will allow to assess cytological alterations associated with findings in MRI studies in all species.

2. Methods

The database Pubmed was searched using a boolean search strategy for each of the 4 aspects of the study while limiting search results to the years 2009-2020. Review articles and studies that did not examine MDD were excluded. Studies from humans, rodents and primates were included, although results concerning primates are rare. Studies were assessed by two raters independently (MA and AM) and a third independent rater (NF) was consulted in case of discrepancies. In the following, specific exclusion criteria for the four aspects of this review are listed.

2.1 MRI Studies in Patients

The keywords `Depression´, ´MRI´, and ´Fractional Anisotropy´ were used to conduct a thorough database search. As the keyword ´White Matter´ would have provided too unspecific and diversified results in studies conducted on patients, the more specific term ´Fractional Anisotropy´ was used. The search results were then limited to studies conducted on humans. This search yielded 365 results. These studies were manually selected to see if they meet any of the exclusion criteria. Exclusion criteria were the following: Patients studied have (I) a psychiatric disorder other than MDD OR (II) a diagnosed neurological disorder OR (III) a documented traumatic brain injury OR (IV) do not meet the age criteria (between 18-65 years of age) OR (V) have received either antidepressant medication or psychotherapy in their lifetime. This resulted in the inclusion of 10 studies and the exclusion of 355 studies. All

included studies obtained informed consent and were carried out in accordance with the Declaration of Helsinki.

2.2 MRI Studies in Rodents and Primates

Since the conduction of MRI studies on primates and rodents is rare, the keywords ´Depression´, ´MRI´, and the more unspecific ´White Matter´ was used. Search results were limited to ´Other Animals´. This yielded 30 results that were manually searched concerning the exclusion criteria. These were the following: (I) animals studied were other than primates, rats or mice OR (II) a disease other than MDD was induced in the animal OR (III) the study did not examine WM alterations OR (IV) the animals received antidepressant medication. This resulted in the inclusion of 6 studies and the exclusion of 24 studies. All included animal experiments complied with the EU Directive 2010/63/EU for animal experiments, or with comparable guidelines for the ethical treatment of animals in research.

2.3 Post-Mortem Studies in Patients

Several studies have investigated the brains of deceased patients who have received a diagnosis of MDD during their lifetime. In order to include these studies, the keywords ´White Matter´, ´Depression´, and ´Post-mortem´ were utilized. Exclusion criteria were the following: the patients (I) did not have a diagnosis of MDD OR (II) had a further psychiatric a neurological disorder OR (III) the study did not investigate WM alterations. This search yielded 54 results, of which 9 were included and 45 excluded. All included studies obtained informed consent were carried out in accordance with the Declaration of Helsinki.

2.4 Post-Mortem Studies in Primates and Rodents

Primates and Rodents provide a suitable model to examine brain alterations in depression on cellular or even on the molecular level. Therefore, a database search was conducted using the keywords ´Depression´ and ´White Matter´, whilst limiting the search results to ´Other Animals´. This yielded 114 results which were manually searched. Exclusion criteria were the following: (I) animals studied were other than rats or mice OR (II) a disease other than MDD was induced in the animal OR (III) the study did not examine WM alterations OR (IV) the animals received antidepressant medication. The application of the exclusion criteria resulted in the inclusion of 9 studies and the exclusion of 105 studies. All included animal experiments complied with the EU Directive 2010/63/EU for animal experiments, or with comparable guidelines for the ethical treatment of animals in research.

3. Findings from MRI Studies

In our review, we are looking at two distinct categories of WM: gyral WM and deep WM. While gyral WM solely consists of the axons belonging to one gyrus, deep WM contains axons from multiple gyri (Rajkowska et al. 2015). All tracts reviewed in the following belong to deep WM.

3.1. Interhemispheric Connectivity

One of the regions displaying replicable significant FA reductions in MDD is the corpus callosum (CC). This structure contains commissural fibers, transmitting information between the two hemispheres. The CC is divided into 4 subregions. These are from rostral to occipital: rostrum, genu, corpus and splenium. Concerning functionality, the forceps minor (FMI) connecting the frontal lobes, as well as the forceps major (FMA) connecting the occipital lobes, can be delineated (Trepel und Dalkowski 2017). Studies conducted on humans have reported a significantly reduced FA in the genu (Sugimoto et al. 2018; Guo et al. 2012) as well as in the FMI (Yang et al. 2017) and the FMA (Won et al. 2017) of the CC in depressive patients, suggesting impaired interhemispheric connectivity between frontal lobes. Furthermore, Cheng et al. could show that early-onset MDD patients (defined as having the first depressive episode before the age of 30; EO) had an increased FA in the CC as well as in the right FMA compared to age-matched healthy controls (HC) (Cheng et al. 2014). Interestingly, late-onset MDD patients (defined as having the first depressive episode above the age of 30; LO) showed no differences in the FA of the CC or the FMA or FMI compared to age-matched HC (Cheng et al. 2014).

Animal studies found alterations concerning the integrity of the CC as well. Zalsman et al. investigated whether Wistar-Kyoto rats (WKY), a depressive and anxious-like breed, show WM alterations compared to control Wistar rats (WIS). In WKY rats, a decreased FA and an increased mean diffusivity (MD) in the CC, as well as decreased FA in the left and right anterior commissures compared to WIS rats were found, indicating impaired interhemispheric frontal connectivity (Zalsman et al. 2017). Of note, despite being neuroanatomically different structures, the CC and the anterior commissure fulfill similar functions, establishing connections between the left and the right brain hemisphere (Zalsman et al. 2017). As the relevant difference between the two groups was the breed they belonged to, it can be assumed that genetic factors are able to cause alterations in WM integrity. Another study investigating the effects of genetic alterations on WM integrity has been carried out by Van der Marel et al., where the influence of a serotonin transporter gene *SLC6A4* knockout was investigated in rats.

In this context, a significantly reduced FA in the genu of the CC of knockout rats was found (van der Marel et al. 2013). Reduced expression of this gene has been linked to depressive disorders both in rodents (Olivier et al. 2008) and in humans (Bleys et al. 2018). These findings show that a defect in the serotonin transporter activity might lead to a depressive phenotype, as well as WM alterations. Furthermore, this study shows that alterations in serotonin homeostasis could be an upstream phenomenon, preceding depressive behavior and impaired connectivity. Thus, alterations in *SLC6A4* expression might be a risk factor for the development of WM alterations.

Besides genetic models of depression, chronic mild stress (CMS) exposure has been shown to cause impaired interhemispheric connectivity in rodents as well. In one study, Hemanth Kumar et al. demonstrated a significant decrease in FA in the CC of Sprague-Dawley rats (Hemanth Kumar et al. 2014). Moreover, animals in the CMS group also exhibited less weight gain and had a lower sucrose intake in the sucrose preference test (SPF), as well as increased immobility in the forced swim test (FST) indicating that the detected impaired interhemispheric connectivity was paralleled by a depressive-like phenotype (Hemanth Kumar et al. 2014). Furthermore, not only human and rodent studies, but also studies on primates have revealed results implicating impaired interhemispheric connectivity in MDD. In a study by Coplan et al., one group of macaques was held under normal conditions, while in the other group mothers had to spend more time foraging due to obstacles placed by the experimenters (Variable Foraging Demand; VFD). Mothers were therewith forced to spend more time away from their offspring which induced stress for the youngsters. This study found that while there was a concordance between the FA of the anterior CC and the anterior limb of the internal capsule (ALIC) in macaques growing up under normal conditions, there was a discordance between these values in VFD macaques (Coplan et al. 2016). In contrast to that, an FA concordance between the posterior limb of the internal capsule (PLIC) and the posterior CC, as well as between occipital WM and the posterior CC could be found in the VFD group, but not in the non-VFD group (Coplan et al. 2016). These findings imply impaired WM integrity in frontal interhemispheric connectivity following early life stress (ELS) and might indicate a disruption in the synchronous development of myelination in frontal WM. Furthermore, they show that ELS is able to disrupt posterior connectivity, causing errant concordance in the WM architecture of this region (Coplan et al. 2016).

From an ontological perspective, the CC shows maturation processes in the postnatal period, mainly in the form of axonal myelination, a process which corresponds to an increase in cognitive functions (Won et al. 2017). Disruptions in this myelination process can negatively

affect working memory, cognition as well as emotional processing (Bae et al. 2006). Such disruptions have been linked to the development of more severe symptoms in MDD (Tham et al. 2011). Moreover, the fact that disruptions in WM integrity were mainly found in rostral regions of the CC points towards the frontal lobes as the structures with the most prominently impaired interhemispheric connectivity. The frontal lobes have been shown to play an important role in MDD, as lesions have been associated with modifications of affect and behavior (Goodwin 1997). Furthermore, they exert a regulatory activity over emotional response, for example, through their connectivity with subcortical regions such as the amygdala (Banks et al. 2007). Frontal lobes also show distinct lateralization concerning their functions. While the left frontal lobe seems to be more strongly involved in cognitive decision making and context-related behavior, the right frontal lobe seems to be critical for tackling challenges posed by novel cognitive situations, as well as for context-independent behavior (Goldberg et al. 1994). Based on these findings, one can hypothesize that impaired connectivity between bilateral frontal lobes could represent alterations in brain circuits responsible for emotion regulation, thus contributing to a depressive phenotype.

While the exact pathological processes concerning alterations of the CC remain unclear, these findings can provide a promising direction for future research. Another aspect that remains to be illuminated is whether these alterations are a cause or a consequence of the disease, thus allowing for a deeper understanding of the pathogenesis of MDD. Taken together, MRI studies investigating WM integrity in the CC provide strong evidence that this region presents impaired architecture in MDD.

3.2 Association Tracts

In addition to assessing commissural fibers, several studies have investigated whether altered diffusion metrics in association tracts, connecting different structures of the same hemisphere, can be detected in patients with MDD, as well as in animal models of depression.

3.2.1 Cingulum Bundle

The cingulum bundle is a highly complex WM tract, connecting the anterior thalamic nuclei, the cingulate gyrus and the parahippocampal region, being part of the so-called Papez circuit (Bubb et al. 2018). This tract contains short and long association fibers, as well as fibers radiating across it, which then aim to reach cortical and subcortical structures (LOCKE et al. 1964). There are only very few connections that run the entire extent of this tract (Heilbronner und Haber 2014). The cingulum bundle is therefore highly sophisticated and diverse, containing fiber tracts associated with different functional entities. Nevertheless, several studies have

investigated the integrity of WM in the cingulum bundle. In this context, one of the reviewed studies has found a significantly decreased FA in the left cingulum of MDD patients (Yang et al. 2017). In rodents, a trend (through a non-significant one) towards a decreased FA in both the left and the right cingulum of WKY rats compared to WIS rats has been found as well (Zalsman et al. 2017). Contradictorily, results indicating increased connectivity have also been reported. In rats that underwent a CMS protocol, a trend towards a non-significant decrease of MD in the bilateral cingulum has been found (Hemanth Kumar et al. 2014). Another study identified a significant increase in FA in mice that underwent a chronic psychosocial stress (CPS) protocol (Grandjean et al. 2016).

The fact that findings concerning the cingulum bundle show conflicting results might be attributed to the versatility of this tract, as well as to the still unclarified role of different segments of this bundle. Therefore, the investigation of this tract might yield divergent results in the same phenotype. Wu et al. established a segmentation of the cingulum bundle in humans using MRI tractography, delineating 5 segments that each connect different brain structures and are parts of distinct functional entities (Wu et al. 2016). These range from the subgenual region playing a critical role in the modulation of negative mood states to parietal regions having cognitive specializations (Wu et al. 2016). Further research that focuses on separately assessing distinct parts of the cingulum could help clarify whether this tract belongs to the brain structures showing unambiguous alterations in MDD.

3.2.2 Longitudinal and Fronto-Occipital Fasciculi

In MDD patients, a decreased FA was found in the left superior longitudinal fasciculus (SLF) (Srivastava et al. 2016) as well as in the right SLF (Wu et al. 2011). Also, such findings were evident in the inferior fronto-occipital fasciculus (IFOF) (Sugimoto et al. 2018) and the left inferior longitudinal fasciculus (ILF) (Won et al. 2017) both showing reduced FA. Moreover, age-related alterations in these tracts could be determined, as EO patients exhibited a decreased FA in the left ILF and an increased FA in the left FOF. Interestingly, the decreased FA in the left ILF could no longer be detected when EO patients with an onset age of 26-29 years were excluded from the calculations. LO patients, on the other hand, expressed a decreased FA in the inferior fronto-occipital fasciculus bilaterally, as well as in the right inferior longitudinal fasciculus (Cheng et al. 2014). In this study, excluding patients close to the delineation of early and late-onset (EO and LO respectively) depression resulted in increased FA in association tracts showing alterations in EO MDD and in decreased FA in tracts showing alterations in LO MDD. Excluding patients close to the delineation makes the differentiation between the two subgroups clearer and thus points to an age-dependent effect. The authors of the study state that

these findings could indicate that EO and LO MDD might be attributable to different etiology and pathophysiology (Cheng et al. 2014).

Studies in animal models of depression investigating association tracts are rare. However, one study investigated alterations in these tracts. Grandjean et al. used CPS in mice to detect aberrant diffusion metrics (Grandjean et al. 2016). To detect changes, measurements from before and after the CPS protocol were compared. Increased functional connectivity in the default mode network of mice in prefrontal and cingulate cortices, as well as in the amygdala-cingulate cortex network was found (Grandjean et al. 2016).

In general, the SLF connects frontal cortical regions with posterior parietal cortical areas, whereby the precise delineation of the origins and terminations of axonal connections are not yet possible (Petrides und Pandya 2012). But as it connects the supplementary motor cortex as well as the premotor cortex and the parietal cortex (Petrides und Pandya 2012), the latter having been linked to spatial attention (Vecera und Rizzo 2003), one can assume a role of this bundle in the integration of environmental influences and responsive motor behavior. The role of impaired connectivity in this fiber bundle in MDD remains to be determined and might require further advances in neuroanatomy and tractography to delineate origins and terminations of axons in this fascicle. However, it should not be neglected that impaired connectivity of this tract seems to be a reproducible finding in MDD patients.

The ILF and the IFOF both run to the occipital lobe, the ILF connecting this region with the temporal lobe, whereas the IFOF connects this area with the frontal cortex, whereby these tracts spatially overlap along a part of their pathway (Ashtari 2012). While the role of these tracts in MDD has not been clarified yet, a lower FA in the ILF in children was correlated with impaired object recognition (Ortibus et al. 2012). As the IFOF and the ILF show overlapping, it has been hypothesized that both tracts likely play a role in object recognition as well (Ashtari 2012). Interestingly, impaired FA in the ILF has also been associated with schizophrenia (Ashtari et al. 2007), as well as with traumatic brain injury and impaired cognitive flexibility (Chanraud et al. 2010).

Alterations in the longitudinal and fronto-occipital fasciculi represent translational findings across rodents and humans. Therefore, it is necessary to further explore and differentiate the functions of these tracts to determine the exact role they play in the pathogenesis of MDD.

3.3 Projection Tracts

Several alterations in projection tracts linking cortical and subcortical structures have been reported. In MDD patients, decreased FA has been found in the anterior corona radiata (Guo et

al. 2012), the internal capsule (Guo et al. 2012; Cheng et al. 2014) as well as the right external capsule (Guo et al. 2012), the right superior thalamic radiation (Cheng et al. 2014) and in the right posterior corona radiate (Cheng et al. 2014). Interestingly, an increased FA has been found in EO patients in the optical radiation as well as in the right corticospinal tract (Cheng et al. 2014). In addition, one of the reviewed studies found a significant negative correlation in LO MDD patients between points reached on the Hamilton Rating Scale for Depression (HAM-RD) and FA in the right anterior and the right posterior corona radiata, the left external capsule and the right ALIC (Cheng et al. 2014), indicating that more severe depression is associated with a more pronounced impairment of WM in these regions. The same study found a positive correlation between FA in the left corticospinal/corticopontine tracts in the mesencephalon and HAM-RD score in EO MDD patients, indicating stronger connectivity in more severe depression (Cheng et al. 2014). A hypothesis to consider in this context is whether this increased connectivity could be affiliated with higher tension of skeletal muscle on the mostly more prominent right side in patients´ bodies. This proposition is supported by the fact that muscle tension has been shown to parallel depression severity (Svebak 1988). Specifically, the corrugator muscles (the mimic muscles that pull the eyebrows downward and medially and are therefore also known as the ´frowning´ muscles) have been shown to have a higher tension in patients with more severe depressive symptoms (Schwartz et al. 1978).

In rodents, an increased MD, indicating reduced connectivity, has been found in the right cerebral peduncle in rats following CMS (Hemanth Kumar et al. 2014). Moreover, reduced FA in the ALIC, but no changes in the PLIC have been reported in male bonnet macaques exposed to VFD (Coplan et al. 2010).

Myelination in the PLIC concludes shortly after birth, myelination of the ALIC, however, is still ongoing in the first postnatal year, a characteristic that is present in both humans and non-human primates (Coplan et al. 2010). Concerning postnatal myelination of the internal capsule, a meta-analysis has found that while myelination in the PLIC can be detected by MRI at the age of 1 month, myelination of the ALIC can only be detected at the age of 2 months in T1- and at the age of 7 months in T2-weighted images (Staudt et al. 2000), indicating ongoing myelination of this region during the first year of development. It has therefore been suggested that this time frame might pose a window of vulnerability, during which the influence of stressors could lead to regional disturbances of myelination (Coplan et al. 2010). While most of the internal capsule contains afferent and efferent fibers connecting the cortex and the spinal cord, the ALIC mostly contains fibers that reciprocally connect the thalamus and the frontal lobes. A bilateral surgical interruption of the thalamocortical radiation arising from the ALIC

has been shown to result in changes to personality, even without damage to the cortex (Freeman und Watts 1942).

In humans, fibers from different frontal-subcortical circuits converge into the internal capsule (Guo et al. 2012). Among these circuits is the orbitofrontal circuit, the dorsolateral prefrontal circuit as well as the anterior cingulate circuit, which have been associated with impaired emotional stability, executive function and motivation, respectively (Guo et al. 2012). The mentioned frontal-subcortical circuits have also been associated with the pathogenesis of MDD (Rogers et al. 1998; Zhu et al. 2011). Reproducible alterations in frontal-subcortical circuits further support the hypothesis that damage to the WM of the internal capsule plays an important role in the development of mood disorders. Based on these translational findings, further research will be required to determine how errant myelination of the ALIC after ELS in humans is involved in the emergence of MDD.

3.4 Gyral White Matter

Concerning gyral WM, FA reductions in the left prefrontal cortex (Srivastava et al. 2016), the left parietal region (Srivastava et al. 2016; Wu et al. 2011), medial frontal gyri (Ouyang et al. 2011), right temporal lobe (Ouyang et al. 2011) and left middle frontal gyrus, as well as cingulate gyrus have been identified in humans (Ouyang et al. 2011). Moreover, Jiang et al. could show correlations between levels of Myelin Oligodendrocyte Glycoprotein (MOG) as well as Myelin Associated Glycoprotein (MAG) in serum as well as FA and MD in the WM of the frontal lobe bilaterally in MDD patients. Such correlations could not be found in healthy subjects (Jiang et al. 2018). MOG and MAG levels in serum were also found to be significantly elevated in MDD patients compared to healthy controls. Although MOG and MAG are relatively minor components of the myelin sheath, they might play an important role in demyelination (Jiang et al. 2018). MAG release, for example, has been shown to be particularly pronounced in early myelination (Jiang et al. 2018). Moreover, demyelination in the context of autoimmune diseases due to antibodies produced against these proteins has been reported as well (Amor et al. 1994). Even though demyelinating diseases, such as multiple sclerosis, are characterized by different symptoms than depression, the findings of demyelination contributing to the pathogenesis should not be discarded in MDD as well. Especially as these findings provide evidence that serum levels of MOG and MAG show correlations with gyral WM integrity in MDD patients.

3.5 Hippocampus

The role of the hippocampus in depression has been extensively studied across rodents and humans. Due to the evolutionally conserved architecture of this structure, studies across different species can yield translatable results. MRI studies in patients with MDD have identified a lower FA in the left hippocampus (Srivastava et al. 2016) as well as a lower pre-treatment FA in the bilateral hippocampus in patients who would turn out to be treatment-resistant, in comparison to those who turned out to be treatment responsive (Zhou et al. 2011). Concerning animal studies, Hemanth Kumar et al. identified a significantly increased axial diffusivity (AD) in the hippocampus of rats subjected to a CMS protocol (Hemanth Kumar et al. 2014). Implying demyelination, a trend towards a higher radial diffusivity (RD) in the left hippocampus was also found, with this result just barely missing significance (Hemanth Kumar et al. 2014).

The hippocampus is generally considered to play a critical role in mood disorders. Evidence in support of this hypothesis is, among others, its involvement in frontal-subcortical circuits (Ongür et al. 2003). This involvement consists of connections formed between the hippocampus, as well as the prefrontal cortex and other subcortical structures, such as the amygdala, thalamic nuclei, and the ventral pallidum (Ongür et al. 2003). This series of linkages is assumed to be crucial in emotional regulation (Ongür et al. 2003). Both increased AD in rats and reduced FA in humans indicate impaired intrahippocampal connectivity, with increased AD pointing specifically towards a loss of axonal density. Whether this impaired connectivity is paralleled by a loss of function of the hippocampus in the circuits mentioned above remains to be determined. A suggestion that has been provided concerning the underlying reason for an increased AD without a significantly increased RD is that a replacement of axonal bundles by glial cells such as astrocytes and microglia might have taken place (Hemanth Kumar et al. 2014), a mechanism that could cause a change in the diffusion profile. Concerning predictability of treatment resistance based on hippocampal FA, it has been hypothesized that lower FA may be caused by hippocampal damage, which could then influence the effects of antidepressant treatment in patients. Neurobiological damage to the hippocampus might, therefore, render patients more prone to being treatment-resistant (Zhou et al. 2011).

Taken together, translational findings show increased diffusivity parameters in the hippocampus across species, indicating structural impairments of this region. While the exact functional implications of these findings remain to be a subject of future research, the fact that the hippocampus expresses reproducible alterations in diffusion metrics might point to the importance this structure plays in the pathogenesis of MDD.

4. Post-Mortem Findings Concerning White Matter Alterations

In the second part of this review, the cellular and molecular correlates of WM alterations will be investigated. Post-mortem studies of WM alterations can be a valuable resource to identify the underlying pathomechanisms of MDD. Studies investigated both, gyral and deep WM, concerning alterations.

4.1 Deep White Matter

Concerning deep WM, studies have not yet yielded satisfactory results to explain alterations identified by MRI. Rajkowska et al. investigated deep WM from the ventral prefrontal cortex in MDD patients who have died from suicide and compared results with patients having died due to a reason other than suicide and without neurological or psychiatric diseases in their lifetime (CTRL). No significant differences between cell density or cell size of Oligodendrocytes (OL) – identified by 2´,3´- Cyclic nucleotide 3´-phosphodiesterase (CNP) immunoreactivity - could be found between MDD and CTRL. Furthermore, no significant differences between OL density and age, or OL density and illness duration could be found. Another aspect that has been investigated in deep WM is the metabolism of polyunsaturated fatty acids (PUFA). These lipids, along with glycerolipids, glycerophospholipids and sphingolipids, play a crucial role in forming cell membranes (Müller et al. 2015). Alterations in the metabolism of these substances have been associated with MDD. Specifically, a diet lacking n-3 PUFA has been shown to induce a depressive phenotype in rodents (Müller et al. 2015). Hamazaki et al. investigated alterations in levels of PUFA in the phospholipid fraction of the CC using thin-layer and gas chromatography. However, the study did not find any significant differences in PUFA levels between MDD Patients and CTRL (Hamazaki et al. 2017). It is important to note that this study only examined relative levels of PUFA, in comparison to other fatty acids (Hamazaki et al. 2017). Whether a reduction in the total amount of PUFA is associated with MDD remains to be determined.

4.2 Gyral White Matter

4.2.1 Oligodendrocyte density and morphology

Several studies have investigated whether reproducible alterations concerning the density and morphology of OL can be identified in gyral WM. A reproducible finding in this context is a reduced density of OL in gyral WM of MDD patients following childhood abuse (CA) (Lutz et al. 2017; Tanti et al. 2018). Of interest, CA is a known risk factor for developing MDD, as reviewed by Carr et al. (Carr et al. 2013). In addition to findings of a reduced OL density, a recent study by Tanti et al. yielded more profound results concerning the influence of CA on

different aspects of OL integrity. CA was defined as severe sexual or physical abuse before the age of 15. Accordingly, 3 groups of patients were defined: suicide victims with depression and CA in their personal history (DS-CA), suicide victims with depression, but without having experienced CA (DS) and control patients, who died of a different reason than suicide and have not had any neurological or psychiatric illnesses during their lifetime (CTRL). As the authors report, DS-CA patients presented a significantly lower density of OL compared to depressed suicide victims who did not experience CA, as well as compared to CTRL (Tanti et al. 2018). OL was identified using immunohistochemistry staining against Olig2, a protein expressed throughout all stages of development by OL (Tanti et al. 2018). In WM, Olig2 is expressed by these cells only (Tanti et al. 2018), making it a specific marker for the whole OL population. Interestingly, no significant difference between DS and CTRL was found, implying that not depression itself, but CA was the driving factor behind altered Olig2+ cell density (Tanti et al. 2018). This finding could be replicated by Lutz et al., who found a significant decrease in total OL number in suicide patients who experienced CA, but not in those who suffered from MDD without experiencing CA (Lutz et al. 2017). Moreover, both studies came to the conclusion, that no difference in the number of oligodendrocyte progenitor cells (OPC) could be detected between the groups (Tanti et al. 2018; Lutz et al. 2017), as identified by the density of cells expressing platelet-derived growth factor receptor α (PDGFRα). Therefore, it has been suggested that the decrease in Olig2+ cells was unrelated to the pool of immature cells (Tanti et al. 2018). Surprisingly, Tanti et al. found a significant increase in the density of mature OL (identified as Nogo-A+ cells) in the DS-CA group, compared to both the DS and the CTRL groups. This result could be confirmed by measuring the density of APC+ cells, another specific marker for mature OL, showing a strong colocalization with Nogo-A (Tanti et al. 2018). Due to the fact that the density of mature OL was significantly increased in DS-CA, while the density of OPC showed no significant group differences, the authors hypothesize that the significant reduction in the total number of OL is caused by a reduction in the number of cells not expressing mature oligodendrocyte markers yet, but also not expressing OPC markers anymore (Tanti et al. 2018). Moreover, an age-related effect of Olig2 expression in DS-CA patients could be identified. There was a significant correlation between age at the time of death and expression of Olig2, meaning older patients showed a higher density of OL than younger patients (Tanti et al. 2018). On the other hand, a significant negative correlation between the number of Nogo-A+ cells and age was found, implying that the number of mature oligodendrocytes decreased with a higher age at suicide (Tanti et al. 2018). The authors, therefore, hypothesize that a recovery of the Olig2+ cell population might take place with

progressing age (Tanti et al. 2018). In order to further investigate the maturation of OL, the authors utilized staining against SOX10. This protein is expressed continuously in OL, though stronger in immature OL than in mature myelinating OL (Tanti et al. 2018). The density of Nogo-A positive OL showing high SOX10 expression, deemed an intermediate phenotype, was significantly lower in DS-CA than in CTRL (Tanti et al. 2018). Taken together, these findings suggest a more mature phenotype of OL in patients having experienced CA. In order to closer examine OL differentiation, staining method against mammalian achaete scute homolog-1 (MASH1), a protein that has been shown to play a critical role in OL maturation and OPC differentiation (Nakatani et al. 2013; Parras et al. 2007) was used. In the DS-CA group, there was a significant increase in MASH1 expression, both compared to DS and CTRL, however no significant difference between DS and CTRL was found (Tanti et al. 2018). Moreover, the MASH1 expression showed a significant negative correlation with age at the time of death, indicating an increased maturation of OL at the age closest to CA (Tanti et al. 2018). This finding implies altered myelination profiles in gyral WM which are specific to CA, but not to MDD. The authors, therefore, hypothesize that CA may trigger a maladaptive increase in the rate of differentiating OL (Tanti et al. 2018).

In a further study, Rajkowska et al. investigated WM alterations in post-mortem brain samples from both suicidal and non-suicidal MDD patients. A positive correlation between OL density and age in the MDD group, however, not in the CTRL group was found (Rajkowska et al. 2015). Moreover, a significant group difference in soma size of OL could be identified, with the MDD group approximately 13% smaller values (Rajkowska et al. 2015). However, no group differences concerning oligodendrocyte density and no further correlations could be shown (Rajkowska et al. 2015).

The reason for these contrasting findings in the studies mentioned above could be that unlike the studies of Tanti et al. and Lutz et al., Rajkowska et al. have not differentiated between depressed patients who experienced CA and those who did not. Based on the studies conducted by Tanti et al. and Lutz et al. we can assume that CA and not MDD is the most important driving force behind alterations of OL density. Therefore, it is possible that in the study conducted by Rajkowska et al., CA patients could not contribute enough to the results to significantly lower the OL density.

4.2.2 Astrocytes

Not only OL but also astrocytes have been investigated concerning their role in MDD. In a study by Torres-Platas et al., it was examined whether depressed suicide patients had altered

astrocyte features, which would be in line with putative disorganization of cortical astrocytic networks (Miguel-Hidalgo et al. 2000). The focus of this study did not lie upon discovering differences in the density of astrocytes between the groups, but instead in characterizing morphologic differences. The authors evaluated two distinct groups of astrocytes: protoplasmic ones, which are found in grey matter (GM), and fibrous ones, which are found in WM. The region of interest was Brodmann´s Area 24, a part of the anterior cingulate cortex (ACC). Compared to control patients, fibrous astrocytes of depressed suicides showed a significantly larger mean cell body size, as well as a more than two-fold increase in the average number of nodes (Torres-Platas et al. 2011). The average number of branch ends, as well as total lengths of astrocyte projections were also significantly higher in depressed suicides than in controls. There were, however, no between-group differences concerning protoplasmic astrocytes (Torres-Platas et al. 2011). The authors also performed a so-called Sholl analysis. In this analysis virtual concentric spheres, which all have their common center in the perikaryon of an astrocyte, are created. This method allows the investigation of the number of intersections, processes, or dendrites per concentric shell. It could be confirmed that processes of fibrous astrocytes had significantly more intersections than controls (Torres-Platas et al. 2011). Process length and branching patterns of fibrous astrocytes were also found to be more elaborate in depressed suicides (Torres-Platas et al. 2011). Taken together, these results imply that astrocytes in the WM of Brodmann´s Area 24 are hypertrophic in depressed suicides, a finding which has been associated with neuroinflammation (Torres-Platas et al. 2011). The correlates of acute astrocyte activation are WM hyperintensities (Torres-Platas et al. 2011), an MRI phenomenon which is not included in our review but has been shown to be associated with depression (Simpson et al. 2007).

In rodents, Gosselin et al. investigated whether WKY rats showed any alterations in astrocytes compared to Sprague-Dawley rats, without using a stress protocol (Gosselin et al. 2009). No significant difference between the density of astrocytes could be found in the CC between the two breeds (Gosselin et al. 2009). In addition, no group differences were found in the density of cells showing S100β expression (Gosselin et al. 2009). This protein is expressed primarily by astrocytes that ensheathe blood vessels, hence creating the blood-brain barrier (Gosselin et al. 2009). It can therefore be assumed that WKY rats do not generally show deviations from Sprague-Dawley rats concerning astrocyte numbers in WM.

4.2.3 Findings on a molecular level

Post-mortem studies allow for a thorough investigation not only of alterations on a cellular level but also on a molecular level. In this context, studies have investigated alterations in protein expression, mRNA expression, as well as DNA damage, both in humans and in rodents.

Concerning alterations in methylation, Lutz et al investigated OL from the ACC of suicide victims. In the study by Lutz et al. mentioned above, a decreased methylation in OL in the DS-CA group compared to the DS group could be identified in the *LINGO3* as well as in the *POU3F1* gene (Lutz et al. 2017). These alterations could not be found in neurons (Lutz et al. 2017). The LINGO3 protein belongs to the LINGO family, a group of proteins that have been linked to myelination (Mi et al. 2005), while POU3F1 is a transcription factor controlling myelination (Ryu et al. 2007). Interestingly, while POU3F1 has been shown to be necessary for myelination (Ryu et al. 2007), LINGO1 seems to negatively influence this process (Mi et al. 2005). Whether LINGO3 has an identical role to LINGO1 remains to be determined. However, these results provide evidence for OL-specific epigenetic alterations, thus further supporting the hypothesis that neither suicide nor MDD, but CA accounts for alterations in myelination. Transcriptomic differences between the groups were also investigated. A total of 32 genes that have been linked to myelination were downregulated in the CA group, while 3 were upregulated (Lutz et al. 2017). Downregulated genes coded for essential building blocks of myelin or were genes that control the synthesis of myelin lipids or were responsible for the differentiation of OL (Lutz et al. 2017). This downregulation was only present in the WM of the ACC, however not in the amygdala (Lutz et al. 2017). This suggests a region-specific impairment of myelination with a focus on frontal areas, which is in line with alterations found in macaques after being exposed to a VFD protocol (Coplan et al. 2016). Despite showing no difference in methylation (Lutz et al. 2017), *ITGB1* mRNA was found to be strongly downregulated in the CA group. Interestingly, the expressions of *LINGO3* and *POU3F1* mRNA were not decreased, despite these genes having been found to be hypermethylated (Lutz et al. 2017). ITGB1 protein promotes myelination by forming complexes with other integrins (ITGA6 and ITGAV), which were also found to be downregulated in the CA group (Lutz et al. 2017). Integrins are crucial for adhesion between cells and extracellular matrix, therefore suggesting that the downregulation of *ITGB1* mRNA is paralleled by an impaired embedding of OL in the surrounding tissue (Lutz et al. 2017). As the downregulation of *ITGB1* mRNA was present in the CA-DS, but not in the DS group, these results suggest that CA is associated with impaired transcription of this essential myelin gene, hence confirming other studies in the finding that not MDD, but CA is closely related to alterations in characteristics of OL. Therefore, future

research should consider the role of altered *ITGB1* expression in patients who experienced CA, in order to clarify its role in altered myelination.

Concerning translational findings, Lutz et al. also evaluated behavior and gene expression in the offspring of dams displaying high or low levels of maternal care, respectively. A strong correlation between myelin gene expression changes in rats raised by low maternal behavior dams and expression changes in humans who experienced CA could be determined (Lutz et al. 2017). The study also used CARS microscopy to further investigate myelination structure. A moderate, but significant decrease in axonal diameter in the CA group compared to both the DS and the CTRL group, could be identified, along with a decrease in myelin thickness in the CA, but not in the DS group. The g-ratio (calculated as the coefficient of axonal thickness and total fiber thickness) was increased in the CA group, meaning that the decrease in myelin thickness outweighed the decrease of the axonal diameter (Lutz et al. 2017), pointing to demyelination being more pronounced than axonal loss. The results suggest that a low level of maternal care in rodents is a suitable translational model to investigate alterations in the expression of myelin related genes in humans having experienced CA. These findings also confirm that childhood adversities may interfere with normal myelination processes across different species.

Findings concerning transcriptomic alterations could be confirmed in other studies. Rajkowska et al. reported a significant decrease in Proteolipid protein 1 (*PLP1*)- mRNA in MDD patients, compared to CTRL. PLP1 is a transmembrane domain protein, which binds copies of itself, thus playing an important role during the wrapping of the myelin sheath. PLP1 defects have been associated with the degeneration of cortical axons in both humans and mice (Garbern et al. 2002). Also, a significant positive correlation between *PLP1* gene expression and OL soma size could be identified, which is in line with other findings of the study reporting a significantly smaller soma size in MDD, without identifying a difference in the OL density (Rajkowska et al. 2015). Although not explicitly identified by this study, the authors speculate that the underlying reason for decreased *PLP1* mRNA expression might be due to PLP1 protein down-regulating mRNA synthesis. On the other hand, a significant up-regulation in the mRNA expression of the OL-enriched genes *CNP*, *MOG* and *Olig1* could be identified in MDD patients. Concerning proteins, CNP protein expression from subjects with MDD was significantly lower than that in controls, despite showing a significantly higher mRNA expression (Rajkowska et al. 2015). This suggests that other procedures than only mRNA expression elicit control over the amount of CNP protein synthesized in OL. Overexpression of *CNP* mRNA has been shown to induce aberrant myelination, leading to accelerated expression of Myelin Basic Protein *(MBP)* and *PLP1* (Gravel et al. 1996), indicating that this alteration

might precede altered *MBP* and *PLP1* expression. Determining the mechanisms that lead to an overexpression of *CNP* mRNA in MDD poses a promising field for future research.

Another aspect that has been investigated in WM is oxidative stress. Szebeni et al. measured levels of 8-Oxo-2´-Deoxyguanosine (8-OXO) as a marker of oxidative stress in the anterior prefrontal cortex of deceased MDD patients. 8-OXO levels were significantly elevated in Brodmann´s Area 10 (part of the medial prefrontal cortex) in MDD donors compared to CTRL (Szebeni et al. 2017). Since many, but not all, MDD patients died of suicide, the authors further investigated whether suicide and oxidative stress show correlations. However, 8-OXO levels were not significantly different in MDD patients that died from suicide compared to MDD patients that died of another reason, indicating that psychiatric illness itself is linked to higher levels of oxidative stress (Szebeni et al. 2017). Moreover, the study investigated the expression of the DNA repair enzymes Poly-ADP-Ribose Polymerase 1 (a DNA damage sensor gene; PARP1) and Oxoguanine Glycosylase 1 (DNA repairing enzyme carrying out the excision of 8-Oxoguanine; OGG1). These enzymes are considered to be markers of oxidative stress in cells (Szebeni et al. 2017). A significantly higher expression of PARP1 and OGG1 in OL of MDD patients could be identified in the uncinate fasciculus (UF) and in Brodmann´s Area 10. Moreover, MDD patients exhibited a significantly increased *PARP1* expression in astrocytes in Brodmann´s Area 10, while no group difference in the *OGG1* expression could be observed in these cells (Szebeni et al. 2017). Of note, neither chronic alcohol consumption nor smoking was found to be associated with differences in DNA oxidation levels, and no significant correlations between length of illness in MDD patients and DNA oxidation levels could be found (Szebeni et al. 2017). The authors hypothesize that oxidative damage might lead to elevated PARP1 activity, thus depleting cellular energy supplies and interfering with important functions of OL (Szebeni et al. 2017). A mechanism that has been proposed in this context is a pro-inflammatory effect of Poly-ADP-Ribose (PAR), which is synthesized by PARP1. Once cleaved from proteins, PAR has been found to trigger an inflammatory response in human and mouse macrophages, acting as an extracellular Damage Associated Molecular Pattern (Krukenberg et al. 2015). Therefore, DNA oxidation might lead to neuroinflammation, which in turn has been repeatedly shown to be associated with depression, as reviewed by Kim et al. (Kim et al. 2016). As chronic stress has been shown to cause significantly higher 8-OXO levels in rats (Szebeni et al. 2017), DNA oxidation could act as a mediator between psychosocial stress and neuroinflammation. In rats, Szebeni et al. also evaluated whether social defeat or unpredictable stressors cause alterations in DNA oxidation. It was determined that this double stress protocol was able to induce anhedonia and reduced social interaction (Szebeni et al. 2017). Moreover, a

significant increase in DNA oxidation in WM, but not in GM could be determined as well (Szebeni et al. 2017). These translational findings suggest that DNA oxidation is linked to depression and stress, and is unlikely to be caused by other confounders in humans (Szebeni et al. 2017).

A further translational study investigated correlations between rodents and humans concerning the role of MicroRNA-21 (MiR-21) in MDD. MiR-21 knockout (KO) mice were found to have a significantly higher PDGFR-α staining in the CC compared to wild type (WT), suggesting an increased number of OPCs (Miguel-Hidalgo et al. 2017). In order to confirm this finding, the authors utilized a staining method against Chondroitin sulfate proteoglycan 4 (CSPG4), another marker of OPCs. There was, however, no significant difference between the amounts of CSPG4 positive cells, so that the exact implications of the increased PDGFR-α staining remain to be determined (Miguel-Hidalgo et al. 2017). The area fraction of MBP immunoreactive fibers in the ACC of miR-21 KO mice was significantly lower compared to WT (Miguel-Hidalgo et al. 2017). Meanwhile in post-mortem samples of human subjects, miR-21 expression (determined by rt-qPCR) was significantly lower in alcoholism, MDD and comorbid alcoholism + MDD than in control subjects in the orbitofrontal cortex (Miguel-Hidalgo et al. 2017). Moreover, there was a significant decrease of *OLIG1* mRNA as well as Glial fibrillary acidic protein *(GFAP)* mRNA in MDD compared to controls in the WM of the orbitofrontal cortex. No such differences were found in alcoholism or in patients suffering from comorbid alcoholism and MDD (Miguel-Hidalgo et al. 2017). While double immunofluorescence determined that MiR-21 is primarily expressed in mature OL, it cannot be ruled out that it could be present in other cells as well (Miguel-Hidalgo et al. 2017). Therefore, the reduced MiR-21 expression that was identified in MDD patients cannot indisputably be linked to OL. MicroRNA plays an important role in gene regulation by binding protein-coding mRNAs and inhibiting their translation (Bushati und Cohen 2007; Valencia-Sanchez et al. 2006; Miguel-Hidalgo et al. 2017). MiR-21 has been mostly linked to carcinomas of the digestive system (Fu et al. 2011), as well as to glioblastomas (Møller et al. 2013), and has only recently been associated with depression, schizophrenia and alcoholism (Miguel-Hidalgo et al. 2017). The exact implication of alterations in miR-21 expression reported in this study is difficult to determine, as various factors other than MicroRNA control the transcription of genes. Whether reductions in miR-21 expression are a cause or a side effect of the alterations in the proteins linked to myelination remains to be determined. However, decreased GFAP and OLIG1 staining in miR-21 KO mice might suggest a pathophysiological role of miR-21 in contributing to alterations determined in astrocyte and OL density, as mentioned previously.

On a molecular level, nodes and paranodes of Ranvier (NoR and PoR, respectively) have been investigated concerning their role in WM alterations in MDD. Miyata et al. found that chronic stress leads to significantly narrower NoR and PoR in the CC of mice (Miyata et al. 2016). The expression of Contactin-associated protein (CASPR) was also investigated. This protein is found in the paranodal region of myelinated axons, between the NoR containing Na^+-Channels and the juxtaparanodal region, containing K^+-Channels (Miyata et al. 2016). CASPR is believed to play a role in intracellular signaling, as well as neuron-glia interaction and it can be used as a marker protein to identify NoR (Miyata et al. 2016). Staining against CASPR showed that areas of CASPR-reactivity were significantly lower in stressed mice, as was the width of NoR, which was found to be reduced by 55% compared to control (Miyata et al. 2016). The expression of Kv1.1, a voltage-gated potassium channel was also investigated. It was found that areas of Kv1.1 immunoreactivity were smaller in chronically stressed mice than in control mice, and that distribution of this channel was significantly more diffuse in stressed animals (Miyata et al. 2016). It was also found that in control mice, CASPR and Kv1.1 were expressed in distinct locations, whereas in stressed mice, the distributions were overlapping in the paranode/juxtaparanode region (Miyata et al. 2016). Furthermore, the study found evidence that chronic stress disrupts normal axon-myelin adhesion, as in chronically stressed mice, Neurofascin was downregulated (Miyata et al. 2016). Neurofascin is a cell adhesion molecule involved in synapse formation and neural development and its deficiency has been associated with disruptions of NoR/PoR complexes, as well as with reduced neural function (Zonta et al. 2008). In accordance with this, Cathomas et al. found that in mice exposed to chronic social stress (CSS), a reduction in the expression of genes for different ion channels could be identified. The mRNA expression of sodium channel type IV beta protein (Scn4b) was strongly downregulated in the basolateral amygdala (BLA). Scn4b is a sodium channel subunit and as such, it is found in the NoR and regulates voltage dependence of sodium channels (Cathomas et al. 2019). Furthermore, the potassium channel subfamily K member 2 (Kcnk2*)*, as well as the adenosine A2a receptor (Adora2a) were found to be downregulated in BLA (Cathomas et al. 2019). Of these proteins, Kcnk2 has been associated with MDD, as distinct single nucleotide polymorphisms in this gene were found significantly more often in patients with MDD than in healthy subjects (Liou et al. 2009). In order to differentiate findings between mature OL and OPC, the authors used immunohistochemistry staining against APC and NG2 respectively. Sholl analysis yielded that mature OL had longer, thicker and more processes in stressed mice than in controls (Miyata et al. 2016). CS did not, however, have an effect on the density or on the morphology of OPC (Miyata et al. 2016). CS also did not lead to microglia activation nor

did it increase the number of astrocytes in the CC (Miyata et al. 2016). Concerning different subtypes of OL, the authors, therefore, conclude that CS has a stronger effect on mature OL than on OPC (Miyata et al. 2016). CS also significantly decreased the Na+/K+ ATPase concentrations and activity in the fiber tract of the CC (Miyata et al. 2016). The Na+/K+ ATPase requires energy in form of ATP in order to function properly. A possibility that should be considered in this context is whether this lower activity could be caused by energy depletion due to DNA oxidation. To identify alterations on a genetic level, the authors used immunocytochemistry. Dexamethasone (DEX) was applied to a cell culture containing mature OL and OPCs. A gene that the authors investigated was the Serum and glucocorticoid-regulated kinase 1 (SGK1). SGK1 is a transcription factor controlling a myriad of cellular functions, including cell proliferation, apoptosis and regulation of cell volume (Miyata et al. 2016). It has gained attention in neuropsychiatric research due to its ability to repress the transcription of the metabotropic glutamate receptors 3 and 5 (mGluR3 and 5) (Miyata et al. 2016). The authors found that the expression of *SGK1* mRNA was significantly increased by DEX stimulation, whereas mGluR3 and -5 mRNA expression was significantly reduced (Miyata et al. 2016). This suggests a decreased OL activity following stress, which could lead to impaired interaction between mature OL and axons (Miyata et al. 2016). Moreover, chronic DEX administration in cell culture led to the formation of more complex and longer OL processes than was the case in OL that were kept under control conditions (Miyata et al. 2016). The formation of myelin-like sheath in cells DEX-treated cells decreased in comparison to control (Miyata et al. 2016). These findings are in line with chronically stressed mice showing similar alterations in OL, as reported in the same study (Miyata et al. 2016). The study also examined patients with MDD. However, as we cannot ensure that patients met this review´s inclusion criteria, this part of the publication is not included here.

Another gene that has been investigated regarding alterations in MDD is *CNP*. Mice that were heterozygous for this gene ($CNP^{+/-}$) were used to test the effects of a mild loss of function of CNP. These mice showed a significant increase in microglia, infiltrating T-lymphocytes as well as astrocytes in the CC, the striatum, and in the anterior commissure (Hagemeyer et al. 2012). In $CNP^{+/-}$ mice, alterations became more profound with increasing age (Hagemeyer et al. 2012). Moreover, an age-dependent increase in neurodegeneration, detected by amyloid precursor protein (APP) staining, was identified. (Hagemeyer et al. 2012) Also, an age-dependent decrease in the expression of *CNP* mRNA was present in WT and $CNP^{+/-}$ mice, but was more prominent in heterozygous mice (Hagemeyer et al. 2012). Findings concerning altered behavior in $CNP^{+/-}$ mice could be confirmed in a study conducted by Cathomas et al. $CNP^{+/-}$ mice showed

a more pronounced activation of microglia, T-lymphocytes and astrocytes, as well as axonal swelling in both GM and WM (Cathomas et al. 2019). Taken together, *CNP*$^{+/-}$ mice seem to show a more pronounced inflammatory phenotype with progressing age, as well as stronger axonal degeneration compared to WT mice. Concerning effects of *CNP*$^{+/-}$ on behavior, different studies utilized several tests, carried out on 24 months old mice. While no significant changes could be found in the OFT between *CNP*$^{+/-}$ and WT mice (Hagemeyer et al. 2012), open arm visits in the elevated plus maze (EPM) were significantly reduced in the *CNP*$^{+/-}$ group (Hagemeyer et al. 2012), meaning heterozygous mice showed normal motor activity and a mildly elevated anxiety profile. *CNP*$^{+/-}$ mice showed reduced social interaction (Hagemeyer et al. 2012; Cathomas et al. 2019) as well as a loss of interest, along with higher floating time in the Morris Water Maze Test (Hagemeyer et al. 2012) and longer immobility time in the TST (Hagemeyer et al. 2012). A catatonic state could also be observed in these mice (Hagemeyer et al. 2012). This was tested by forcing mice to stand on their hind legs while grabbing a bar. WT mice left this position swiftly, whilst *CNP*$^{+/-}$ mice persisted in this posture for a significantly longer amount of time (Hagemeyer et al. 2012). This has been interpreted by the authors as an equivalent to a catatonia-like behavior (Hagemeyer et al. 2012). The fact that the alterations in *CNP*$^{+/-}$ mice were found to be age-related might indicate that heterozygosity for this gene poses a vulnerability factor for alterations, which, however, still requires a second hit (e.g. aging) in order to unfold its effects (Hagemeyer et al. 2012). This phenomenon is supported by human studies, which found that elderly patients with MDD are more likely to present with symptoms of catatonic depression, whereas these symptoms are absent in virtually all young patients (Hagemeyer et al. 2012). Cathomas et al. also studied the expression of OL-related genes. Gene expression was evaluated using rt-PCR on samples obtained from the ventromedial prefrontal cortex, the BLA as well as the central nucleus of the amygdala (CeA). In the ventromedial prefrontal cortex, genes for MBP and Myelin-associated Oligodendrocyte Basic Protein (MOBP) showed reduced mRNA expression, whereas such differences could not be detected in MOG, MAG and PLP1 mRNA (Cathomas et al. 2019). In the BLA, expression of the genes coding for MBP, MOBP and CNP1 were downregulated (Cathomas et al. 2019). The CeA demonstrated decreased mRNA expression of MBP and MOBP (Cathomas et al. 2019). Moreover, a 20% decrease in the OL population of the BLA was identified (Cathomas et al. 2019). It is important to note that this study did not specifically look at WM, but instead took samples from the brain regions mentioned above and therefore analyzed both GM and WM in these regions.

Several further studies could confirm that stress and a depressive phenotype lead to WM alterations in rodents. In this context, a significant reduction of MBP in rats expressing a depressive-like phenotype could be identified (Gao et al. 2017). Moreover, after being exposed to different stress protocols, a decrease in total WM volume (Gao et al. 2017; Xiao et al. 2018; Chen et al. 2016), as well as in total length (Xiao et al. 2018; Gao et al. 2017), total volume (Xiao et al. 2018) and mean diameter of myelinated fibers (Xiao et al. 2018; Gao et al. 2017), as well as decreased total volume and thickness of myelin sheath, could be identified in rats (Xiao et al. 2018). Moreover, shorter total capillary length, lower total capillary volume and smaller total capillary surface area after CUS could be identified in rats (Chen et al. 2016).

An aspect that has recently gained attention is whether WM alterations can be reversed using exercise. In this context, running exercise daily for 4 weeks could be repeatedly shown to reduce depressive-like behavior (Chen et al. 2016; Xiao et al. 2018). Moreover, rats that underwent a 4-week running exercise protocol did not significantly differ from control animals concerning total WM volume (Xiao et al. 2018; Chen et al. 2016), total length of myelinated axons (Xiao et al. 2018), total volume of myelinated fibers (Xiao et al. 2018), total volume and thickness of myelin sheath (Xiao et al. 2018) as well as total capillary length in WM (Chen et al. 2016). It is important to note that in these experiments exercise led to an absence of WM alterations despite being utilized after exposure to stress. In contrast to that, antidepressants could until now only be shown to lead to an absence of WM alterations if applied simultaneously with stress (Wang et al. 2014; Abdel-Wahab und Salama 2011), however, not if applied afterwards (Gao et al. 2019). These results show that further research concerning the therapeutic and preventive properties of exercise and antidepressant medication is required in order to utilize these methods to their fullest capacities.

5. Discussion

In the present review, we evaluated translational findings concerning WM alterations in depression identified using MRI and post-mortem samples of human and animal brains. Imaging studies provide strong evidence that interhemispheric and intrahemispheric connectivity, as well as connectivity between cortical and subcortical regions, are altered in depression. Several tracts show increased connectivity, while the majority shows imaging correlates of decreased connectivity. The most stable finding in treatment-naïve humans with MDD is a disruption in the WM integrity of rostral regions of the CC (Sugimoto et al. 2018; Guo et al. 2012; Yang et al. 2017; Won et al. 2017). Imaging studies conducted on macaques exposed to ELS could confirm aberrant myelination in the ALIC (Coplan et al. 2016), a region

which is myelinated postnatally in macaques (Coplan et al. 2016) as well as in humans (Staudt et al. 2000), thus providing evidence for alterations in the myelination of areas that are subject to maturation during early life. Moreover, rodent studies could confirm that both, genetic alterations that have been associated with a depressive phenotype (van der Marel et al. 2013; Zalsman et al. 2017), as well as chronic stress (Hemanth Kumar et al. 2014), were able to induce comparable neuroimaging alterations to those found in humans suffering from MDD. Translational studies provide strong evidence for the frontal regions being the focus of neuroimaging alterations in depressive phenotypes across a range of mammalian species. Studies in animal models of depression can help eliminate confounders such as smoking and alcohol consumption that are an inevitable element of human studies, thus allowing for a more precise evaluation of underlying processes leading to alterations. Further longitudinal studies in rodents concerning age-related neuroimaging correlates of WM alterations in depression could shed light upon the pathomechanism of impaired WM integrity.

By evaluating post-mortem samples of humans, primates and rodents, cellular and molecular correlates of impaired connectivity could be determined. In humans having experienced CA, a decrease in total OL density in gyral WM could be determined (Lutz et al. 2017; Tanti et al. 2018), with a shift towards a more mature phenotype of OL (Tanti et al. 2018). These alterations were present in victims of CA, however, not in MDD patients who did not experience CA (Tanti et al. 2018). This suggests that alterations in the characteristics of OL might not directly be linked to MDD but could rather represent a consequence of ELS. Nevertheless, since ELS has repeatedly been shown to pose a risk factor for psychiatric disease (Carr et al. 2013), the role of altered characteristics of OL following CA should not be discarded as a possible pathomechanism leading up to MDD. Therefore, studies need to separate MDD patients having experienced CA from patients without an experience of CA, thus allowing for a more sophisticated analysis.

In this review, we could also provide evidence for age-dependent alterations in myelination in both, neuroimaging and post-mortem studies. Especially in MDD patients having experienced CA, results show more pronounced maturation of OL in years closer to the CA experience (Tanti et al. 2018). Since the total density of OL is decreased, while the density of mature OL is increased in these patients, a possible mechanism that needs consideration as a consequence of CA, is demyelination. In support of this, Jiang identified increased MOG and MAG serum levels, indicating demyelination, in depressive patients (Jiang et al. 2018). While typical demyelinating diseases- such as multiple sclerosis- present with different symptoms than MDD, these diseases show a strong association with depression, as reviewed by Siegert et al. (Siegert

und Abernethy 2005). A possible overlap in the pathomechanism of multiple sclerosis and depression, therefore, cannot be discarded.

Not only demyelination but also insufficient myelin synthesis is a possible mechanism leading to WM alterations. In this context, DNA oxidation has been suggested as the link between stress and aberrant myelination (Szebeni et al. 2017). Stress has been shown to cause DNA oxidation, which is known to deplete intracellular energy reserves (Szebeni et al. 2017). Energy depletion leads to intracellular alterations that inhibit the HMG-CoA-Reductase, thus reducing the production of cholesterol, one of the most important building blocks of myelin (Saher et al. 2005). Further evidence in support of this hypothesis is neuroinflammation, a process that has been shown to be a consequence of DNA oxidation and has also been identified in the context of MDD. DNA oxidation as a consequence of stress has been shown in mice, as well as in deceased MDD patients (Szebeni et al. 2017). CA, as a stressor, could cause DNA oxidation, which could then ultimately lead to decreased myelination. This hypothesis is in line with macaques exposed to ELS showing decreased myelination in postnatally maturing brain areas, but not in areas where the maturation is completed prenatally (Coplan et al. 2016). Since frontal areas show a postnatal maturation in humans as well (Staudt et al. 2000), this mechanism might also be present in MDD patients having suffered CA. Assessment of neuroimaging differences between CA and non-CA MDD patients could, therefore, pose a promising field of future research.

The development of neuroimaging biomarkers of depression currently poses an important field of research. Identifying such a biomarker could ease the work of clinicians, providing a tool to distinguish between different psychiatric disorders. As presented in our review, many alterations in diffusion metrics are inconsistent and remain to be reliably replicated. FA reductions in frontal areas of the CC might, however, represent such a finding. So far, FA reductions in the genu of the CC have been found to be absent in several neurologic and psychiatric diseases, such as in unmedicated patients suffering from schizophrenia (Gasparotti et al. 2009) or multiple sclerosis (Hasan et al. 2005). However, this finding does not seem to be entirely specific to MDD, as it has been identified in migraine (Yuan et al. 2012) as well as in bipolar disorder (Wang et al. 2008). Due to the fact that there is currently no unequivocal WM alteration that is specific to MDD, this neuroimaging method is not yet suitable to serve as a biomarker for depression. Further studies with more refined criteria are required to identify potential subgroups of patients expressing reproducible WM alterations.

To conclude, neuroimaging studies, as well as post-mortem examinations, point to an important role of WM in MDD. Alterations in inter- and intrahemispheric communication might have a vast impact on cognitive and emotional behavior, thus leading to deficits observed in MDD patients. Preventing WM alterations early on might prove a promising step towards reducing symptom severity and disability. Animal models of depression show similar alterations and thus might help the study of potential pharmacological targets. Moreover, studies investigating longitudinal WM changes in animals could provide a valuable resource to assess the age-dependence of WM alterations. A combination of these studies could provide important information to entirely unravel the mystery of WM alterations.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

The manuscript was written by M.A., A.M. and N.F. All authors approved the manuscript.

Abbreviations

8-OXO: 8-Oxo-2´-Deoxyguanosine
ACC: Anterior Cingulate Cortex
AD: Axial Diffusivity
Adora2a: Adenosine A2a Receptor
ALIC: Anterior Limb of the Internal Capsule
BLA: Basolateral Amygdala
CA: Childhood Abuse
CASPR: Contactin-Associated Protein
CC: Corpus Callosum
CeA: Central Nucleus of the Amygdala
CMS: Chronic Mild Stress
CNP: 2´,3´- Cyclic nucleotide 3´-phosphodiesterase
CNS: Central Nervous System
CPS: Chronic Psychosocial Stress
CSPG4: Chodroitin Sulfate Proteoglycan 4
CSS: Chronic Social Stress
CTRL: Control Patients
CUS: Chronic Unpredictable Stress
DEX: Dexamethasone
DS: Suicide Victims with Depression
DS-CA: Suicide Victims with Depression and Childhood Abuse
ELS: Early Life Stress
EO: Early-Onset
FA: Fractional Anisotropy
FMA: Forceps Major
FMI: Forceps Minor
FST: Forced Swim Test
GFAP: Glial Fibrillary Acidic Protein

GM: Gray Matter
HAM-RD: Hamilton Rating Scale for Depression
HC: Healthy Controls
IFOF: Inferior Fronto-Occipital Fasciculus
ILF: Inferior Longitudinal Fasciculus
Kcnk2: potassium channel subfamily K member 2
LO: Late-Onset
MAG: Myelin Associated Glycoprotein
MASH1: mammalian achaete scute homolog-1
MD: Mean Diffusivity
MDD: Major Depressive Disorder
mGluR: Metabotropic Glutamate Receptor
MiR-21: Micro-RNA 21
MOBP: Myelin-Associated Oligodendrocyte Basic Protein
MOG: Myelin Oligodendrocyte Glycoprotein
MRI: Magnet Resonance Imaging
NoR: Node of Ranvier
OL: Oligodendrocyte
OPC: Oligodendrocyte Progenitor Cells
PAR: Poly-ADP-Ribose
PDGFRα: platelet-derived growth factor receptor α
PLIC: Posterior Limb of the Internal Capsule
PLP1: Proteolipid protein 1
PoR: Paranode of Ranvier
PUFA: Polyunsaturated Fatty Acids
RD: Radial Diffusivity
Scn4b: sodium channel type IV beta protein
SGK1: Serum and Glucocorticoid-Regulated Kinase 1
SLF: Superior Longitudinal Fasciculus
SPF: Sucrose Preference Test
UCMS: Unpredictable Chronic Mild Stress Protocol
VFD: Variable Foraging Demand
WIS: Wistar
WKY: Wistar-Kyoto
WM: White Matter
YLD: Years Lost to Disability

References

Abdel-Wahab, Basel A.; Salama, Ragaa H. (2011): Venlafaxine protects against stress-induced oxidative DNA damage in hippocampus during antidepressant testing in mice. In: *Pharmacology, biochemistry, and behavior* 100 (1), S. 59–65. DOI: 10.1016/j.pbb.2011.07.015.

Amor, S.; Groome, N.; Linington, C.; Morris, M. M.; Dornmair, K.; Gardinier, M. V. et al. (1994): Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. In: *J Immunol* 153 (10), S. 4349–4356. Online verfügbar unter https://research.vumc.nl/en/publications/identification-of-epitopes-of-myelin-oligodendrocyte-glycoprotein.

Ashtari, Manzar (2012): Anatomy and functional role of the inferior longitudinal fasciculus: a search that has just begun. In: *Developmental Medicine & Child Neurology* 54 (1), S. 6–7. DOI: 10.1111/j.1469-8749.2011.04122.x.

Ashtari, Manzar; Cottone, John; Ardekani, Babak A.; Cervellione, Kelly; Szeszko, Philip R.; Wu, Jinghui et al. (2007): Disruption of white matter integrity in the inferior longitudinal fasciculus in adolescents with schizophrenia as revealed by fiber tractography. In: *Arch Gen Psychiatry* 64 (11), S. 1270–1280. DOI: 10.1001/archpsyc.64.11.1270.

Bae, Jae Nam; MacFall, James R.; Krishnan, K. Ranga R.; Payne, Martha E.; Steffens, David C.; Taylor, Warren D. (2006): Dorsolateral prefrontal cortex and anterior cingulate cortex white matter alterations in late-life depression. In: *Biological Psychiatry* 60 (12), S. 1356–1363. DOI: 10.1016/j.biopsych.2006.03.052.

Baerwald, C.; Manger, B.; Hueber, A. (2019): Depression als Komorbidität bei rheumatoider Arthritis. In: *Zeitschrift fur Rheumatologie* 78 (3), S. 243–248. DOI: 10.1007/s00393-018-0568-5.

Banks, Sarah J.; Eddy, Kamryn T.; Angstadt, Mike; Nathan, Pradeep J.; Phan, K. Luan (2007): Amygdala-frontal connectivity during emotion regulation. In: *Social cognitive and affective neuroscience* 2 (4), S. 303–312. DOI: 10.1093/scan/nsm029.

Bleys, Dries; Luyten, Patrick; Soenens, Bart; Claes, Stephan (2018): Gene-environment interactions between stress and 5-HTTLPR in depression: A meta-analytic update. In: *Journal of affective disorders* 226, S. 339–345. DOI: 10.1016/j.jad.2017.09.050.

Bubb, Emma J.; Metzler-Baddeley, Claudia; Aggleton, John P. (2018): The cingulum bundle: Anatomy, function, and dysfunction. In: *Neuroscience and biobehavioral reviews* 92, S. 104–127. DOI: 10.1016/j.neubiorev.2018.05.008.

Bushati, Natascha; Cohen, Stephen M. (2007): microRNA functions. In: *Annual review of cell and developmental biology* 23, S. 175–205. DOI: 10.1146/annurev.cellbio.23.090506.123406.

Carr, Clara Passmann; Martins, Camilla Maria Severi; Stingel, Ana Maria; Lemgruber, Vera Braga; Juruena, Mario Francisco (2013): The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. In: *The Journal of nervous and mental disease* 201 (12), S. 1007–1020. DOI: 10.1097/NMD.0000000000000049.

Cathomas, F.; Azzinnari, D.; Bergamini, G.; Sigrist, H.; Buerge, M.; Hoop, V. et al. (2019): Oligodendrocyte gene expression is reduced by and influences effects of chronic social stress in mice. In: *Genes, brain, and behavior* 18 (1), e12475. DOI: 10.1111/gbb.12475.

Chanraud, Sandra; Zahr, Natalie; Sullivan, Edith V.; Pfefferbaum, Adolf (2010): MR diffusion tensor imaging: a window into white matter integrity of the working brain. In: *Neuropsychology review* 20 (2), S. 209–225. DOI: 10.1007/s11065-010-9129-7.

Chen, Lin-Mu; Zhang, Ai-Pin; Wang, Fei-Fei; Tan, Chuan-Xue; Gao, Yuan; Huang, Chun-Xia et al. (2016): Running exercise protects the capillaries in white matter in a rat model of depression. In: *The Journal of comparative neurology* 524 (17), S. 3577–3586. DOI: 10.1002/cne.24017.

Cheng, Yuqi; Xu, Jian; Yu, Hongjun; Nie, Binbin; Li, Na; Luo, Chunrong et al. (2014): Delineation of early and later adult onset depression by diffusion tensor imaging. In: *PloS one* 9 (11), e112307. DOI: 10.1371/journal.pone.0112307.

Coplan, Jeremy D.; Abdallah, Chadi G.; Tang, Cheuk Y.; Mathew, Sanjay J.; Martinez, Jose; Hof, Patrick R. et al. (2010): The role of early life stress in development of the anterior limb of the internal capsule in nonhuman primates. In: *Neuroscience letters* 480 (2), S. 93–96. DOI: 10.1016/j.neulet.2010.06.012.

Coplan, Jeremy D.; Kolavennu, Venu; Abdallah, Chadi G.; Mathew, Sanjay J.; Perera, Tarique D.; Pantol, Gustavo et al. (2016): Patterns of anterior versus posterior white matter fractional

anistotropy concordance in adult nonhuman primates: Effects of early life stress. In: *Journal of affective disorders* 192, S. 167–175. DOI: 10.1016/j.jad.2015.11.049.

Currie, Shawn R.; Wang, Jianli (2005): More data on major depression as an antecedent risk factor for first onset of chronic back pain. In: *Psychological medicine* 35 (9), S. 1275–1282. DOI: 10.1017/S0033291705004952.

Eaton, W. W.; Armenian, H.; Gallo, J.; Pratt, L.; Ford, D. E. (1996): Depression and risk for onset of type II diabetes. A prospective population-based study. In: *Diabetes care* 19 (10), S. 1097–1102. DOI: 10.2337/diacare.19.10.1097.

Edgar, N.; Sibille, E. (2012): A putative functional role for oligodendrocytes in mood regulation. In: *Transl Psychiatry* 2 (5), e109. DOI: 10.1038/tp.2012.34.

Fava, Maurizio; Rankin, Meridith A.; Wright, Emma C.; Alpert, Jonathan E.; Nierenberg, Andrew A.; Pava, Joel; Rosenbaum, Jerrold F. (2000): Anxiety disorders in major depression. In: *Comprehensive Psychiatry* 41 (2), S. 97–102. DOI: 10.1016/S0010-440X(00)90140-8.

Freeman, W.; Watts, J. W. (1942): Prefrontal Lobotomy: The Surgical Relief of Mental Pain. In: *Bulletin of the New York Academy of Medicine* 18 (12), S. 794–812.

Fu, Xiaonan; Han, Yijie; Wu, Ying; Zhu, Xiaoli; Lu, Xin; Mao, Feng et al. (2011): Prognostic role of microRNA-21 in various carcinomas: a systematic review and meta-analysis. In: *European Journal of Clinical Investigation* 41 (11), S. 1245–1253. DOI: 10.1111/j.1365-2362.2011.02535.x.

Gao, Yuan; Ma, Jing; Tang, Jing; Liang, Xin; Huang, Chun-Xia; Wang, San-Rong et al. (2017): White matter atrophy and myelinated fiber disruption in a rat model of depression. In: *The Journal of comparative neurology* 525 (8), S. 1922–1933. DOI: 10.1002/cne.24178.

Gao, Yuan; Yao, Yao; Liang, Xin; Tang, Jing; Ma, Jing; Qi, Ying-Qiang et al. (2019): Changes in white matter and the effects of fluoxetine on such changes in the CUS rat model of depression. In: *Neuroscience letters* 694, S. 104–110. DOI: 10.1016/j.neulet.2018.11.013.

Garbern, James Y.; Yool, Donald A.; Moore, Gregory J.; Wilds, Ian B.; Faulk, Michael W.; Klugmann, Matthias et al. (2002): Patients lacking the major CNS myelin protein, proteolipid protein 1, develop length-dependent axonal degeneration in the absence of demyelination and inflammation. In: *Brain* 125 (Pt 3), S. 551–561. DOI: 10.1093/brain/awf043.

Gasparotti, Roberto; Valsecchi, Paolo; Carletti, Francesco; Galluzzo, Alessandro; Liserre, Roberto; Cesana, Bruno; Sacchetti, Emilio (2009): Reduced fractional anisotropy of corpus callosum in first-contact, antipsychotic drug-naive patients with schizophrenia. In: *Schizophrenia Research* 108 (1-3), S. 41–48. DOI: 10.1016/j.schres.2008.11.015.

Goldberg, E.; Podell, K.; Lovell, M. (1994): Lateralization of frontal lobe functions and cognitive novelty. In: *The Journal of neuropsychiatry and clinical neurosciences* 6 (4), S. 371–378. DOI: 10.1176/jnp.6.4.371.

Goodwin, G. M. (1997): Neuropsychological and neuroimaging evidence for the involvement of the frontal lobes in depression. In: *Journal of psychopharmacology (Oxford, England)* 11 (2), S. 115–122. DOI: 10.1177/026988119701100204.

Gosselin, R-D; Gibney, S.; O'Malley, D.; Dinan, T. G.; Cryan, J. F. (2009): Region specific decrease in glial fibrillary acidic protein immunoreactivity in the brain of a rat model of depression. In: *Neuroscience* 159 (2), S. 915–925. DOI: 10.1016/j.neuroscience.2008.10.018.

Grandjean, Joanes; Azzinnari, Damiano; Seuwen, Aline; Sigrist, Hannes; Seifritz, Erich; Pryce, Christopher R.; Rudin, Markus (2016): Chronic psychosocial stress in mice leads to changes in

brain functional connectivity and metabolite levels comparable to human depression. In: *NeuroImage* 142, S. 544–552. DOI: 10.1016/j.neuroimage.2016.08.013.

Gravel, M.; Peterson, J.; Yong, V. W.; Kottis, V.; Trapp, B.; Braun, P. E. (1996): Overexpression of 2',3'-cyclic nucleotide 3'-phosphodiesterase in transgenic mice alters oligodendrocyte development and produces aberrant myelination. In: *Molecular and cellular neurosciences* 7 (6), S. 453–466. DOI: 10.1006/mcne.1996.0033.

Guo, Wen-bin; Liu, Feng; Xue, Zhi-min; Gao, Keming; Wu, Ren-rong; Ma, Chao-qiong et al. (2012): Altered white matter integrity in young adults with first-episode, treatment-naive, and treatment-responsive depression. In: *Neuroscience letters* 522 (2), S. 139–144. DOI: 10.1016/j.neulet.2012.06.027.

Hagemeyer, Nora; Goebbels, Sandra; Papiol, Sergi; Kästner, Anne; Hofer, Sabine; Begemann, Martin et al. (2012): A myelin gene causative of a catatonia-depression syndrome upon aging. In: *EMBO molecular medicine* 4 (6), S. 528–539. DOI: 10.1002/emmm.201200230.

Hamazaki, K.; Maekawa, M.; Toyota, T.; Dean, B.; Hamazaki, T.; Yoshikawa, T. (2017): Fatty acid composition of the postmortem corpus callosum of patients with schizophrenia, bipolar disorder, or major depressive disorder. In: *European psychiatry : the journal of the Association of European Psychiatrists* 39, S. 51–56. DOI: 10.1016/j.eurpsy.2016.05.007.

Hasan, Khader M.; Gupta, Rakesh K.; Santos, Rafael M.; Wolinsky, Jerry S.; Narayana, Ponnada A. (2005): Diffusion tensor fractional anisotropy of the normal-appearing seven segments of the corpus callosum in healthy adults and relapsing-remitting multiple sclerosis patients. In: *Journal of magnetic resonance imaging : JMRI* 21 (6), S. 735–743. DOI: 10.1002/jmri.20296.

Heilbronner, Sarah R.; Haber, Suzanne N. (2014): Frontal cortical and subcortical projections provide a basis for segmenting the cingulum bundle: implications for neuroimaging and psychiatric disorders. In: *J. Neurosci.* 34 (30), S. 10041–10054. DOI: 10.1523/JNEUROSCI.5459-13.2014.

Hemanth Kumar, B. S.; Mishra, S. K.; Trivedi, R.; Singh, S.; Rana, P.; Khushu, S. (2014): Demyelinating evidences in CMS rat model of depression: a DTI study at 7 T. In: *Neuroscience* 275, S. 12–21. DOI: 10.1016/j.neuroscience.2014.05.037.

James, Spencer L.; Abate, Degu; Abate, Kalkidan Hassen; Abay, Solomon M.; Abbafati, Cristiana; Abbasi, Nooshin et al. (2018): Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. In: *The Lancet* 392 (10159), S. 1789–1858. DOI: 10.1016/S0140-6736(18)32279-7.

Jiang, Linling; Cheng, Yuqi; Jiang, Hongyan; Xu, Jian; Lu, Jin; Shen, Zonglin et al. (2018): Association between abnormal serum myelin-specific protein levels and white matter integrity in first-episode and drug-naïve patients with major depressive disorder. In: *Journal of affective disorders* 232, S. 61–68. DOI: 10.1016/j.jad.2018.02.044.

Kathol, R. G.; Jaeckle, R. S.; Lopez, J. F.; Meller, W. H. (1989): Pathophysiology of HPA axis abnormalities in patients with major depression: an update. In: *The American journal of psychiatry* 146 (3), S. 311–317. DOI: 10.1176/ajp.146.3.311.

Kim, Yong-Ku; Na, Kyoung-Sae; Myint, Aye-Mu; Leonard, Brian E. (2016): The role of pro-inflammatory cytokines in neuroinflammation, neurogenesis and the neuroendocrine system in major depression. In: *Progress in neuro-psychopharmacology & biological psychiatry* 64, S. 277–284. DOI: 10.1016/j.pnpbp.2015.06.008.

Krukenberg, Kristin A.; Kim, Sujeong; Tan, Edwin S.; Maliga, Zoltan; Mitchison, Timothy J. (2015): Extracellular poly(ADP-ribose) is a pro-inflammatory signal for macrophages. In: *Chemistry & biology* 22 (4), S. 446–452. DOI: 10.1016/j.chembiol.2015.03.007.

Liou, Ying-Jay; Chen, Tai-Jui; Tsai, Shih-Jen; Yu, Younger W-Y; Cheng, Chih-Ya; Hong, Chen-Jee (2009): Support for the involvement of the KCNK2 gene in major depressive disorder and response to antidepressant treatment. In: *Pharmacogenetics and genomics* 19 (10), S. 735–741. DOI: 10.1097/FPC.0b013e32832cbe61.

LOCKE, S.; ANGEVINE, J. B.; YAKOVLEV, P. I. (1964): LIMBIC NUCLEI OF THALAMUS AND CONNECTIONS OF LIMBIC CORTEX. VI. THALAMOCORTICAL PROJECTION OF LATERAL DORSAL NUCLEUS IN CAT AND MONKEY. In: *Arch Neurol* 11 (1), S. 1–12. DOI: 10.1001/archneur.1964.00460190005001.

Lutz, Pierre-Eric; Tanti, Arnaud; Gasecka, Alicja; Barnett-Burns, Sarah; Kim, John J.; Zhou, Yi et al. (2017): Association of a History of Child Abuse With Impaired Myelination in the Anterior Cingulate Cortex: Convergent Epigenetic, Transcriptional, and Morphological Evidence. In: *The American journal of psychiatry* 174 (12), S. 1185–1194. DOI: 10.1176/appi.ajp.2017.16111286.

McNamara, Robert K.; Lotrich, Francis E. (2012): Elevated immune-inflammatory signaling in mood disorders: a new therapeutic target? In: *Expert review of neurotherapeutics* 12 (9), S. 1143–1161. DOI: 10.1586/ern.12.98.

Mi, Sha; Miller, Robert H.; Lee, Xinhua; Scott, Martin L.; Shulag-Morskaya, Svetlane; Shao, Zhaohui et al. (2005): LINGO-1 negatively regulates myelination by oligodendrocytes. In: *Nat Neurosci* 8 (6), S. 745–751. DOI: 10.1038/nn1460.

Miguel-Hidalgo, José Javier; Baucom, Christie; Dilley, Ginny; Overholser, James C.; Meltzer, Herbert Y.; Stockmeier, Craig A.; Rajkowska, Grazyna (2000): Glial fibrillary acidic protein immunoreactivity in the prefrontal cortex distinguishes younger from older adults in major depressive disorder. In: *Biological Psychiatry* 48 (8), S. 861–873. DOI: 10.1016/S0006-3223(00)00999-9.

Miguel-Hidalgo, José Javier; Hall, Katherine O.; Bonner, Hannah; Roller, Anna M.; Syed, Maryam; Park, Casey J. et al. (2017): MicroRNA-21: Expression in oligodendrocytes and correlation with low myelin mRNAs in depression and alcoholism. In: *Progress in neuro-psychopharmacology & biological psychiatry* 79 (Pt B), S. 503–514. DOI: 10.1016/j.pnpbp.2017.08.009.

Miyata, Shingo; Taniguchi, Manabu; Koyama, Yoshihisa; Shimizu, Shoko; Tanaka, Takashi; Yasuno, Fumihiko et al. (2016): Association between chronic stress-induced structural abnormalities in Ranvier nodes and reduced oligodendrocyte activity in major depression. In: *Scientific reports* 6, S. 23084. DOI: 10.1038/srep23084.

Møller, Heidi G.; Rasmussen, Andreas P.; Andersen, Hjalte H.; Johnsen, Kasper B.; Henriksen, Michael; Duroux, Meg (2013): A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion. In: *Mol Neurobiol* 47 (1), S. 131–144. DOI: 10.1007/s12035-012-8349-7.

Müller, Christian P.; Reichel, Martin; Mühle, Christiane; Rhein, Cosima; Gulbins, Erich; Kornhuber, Johannes (2015): Brain membrane lipids in major depression and anxiety disorders. In: *Biochimica et biophysica acta* 1851 (8), S. 1052–1065. DOI: 10.1016/j.bbalip.2014.12.014.

Nakatani, Hiroko; Martin, Elodie; Hassani, Hessameh; Clavairoly, Adrien; Maire, Cécile L.; Viadieu, Arthur et al. (2013): Ascl1/Mash1 promotes brain oligodendrogenesis during

myelination and remyelination. In: *J. Neurosci.* 33 (23), S. 9752–9768. DOI: 10.1523/JNEUROSCI.0805-13.2013.

Olivier, J. D. A.; van der Hart, M. G. C.; van Swelm, R. P. L.; Dederen, P. J.; Homberg, J. R.; Cremers, T. et al. (2008): A study in male and female 5-HT transporter knockout rats: an animal model for anxiety and depression disorders. In: *Neuroscience* 152 (3), S. 573–584. DOI: 10.1016/j.neuroscience.2007.12.032.

Ongür, Dost; Ferry, Amon T.; Price, Joseph L. (2003): Architectonic subdivision of the human orbital and medial prefrontal cortex. In: *The Journal of comparative neurology* 460 (3), S. 425–449. DOI: 10.1002/cne.10609.

Ortibus, Els; Verhoeven, Judith; Sunaert, Stefan; Casteels, Ingele; Cock, Paul de; Lagae, Lieven (2012): Integrity of the inferior longitudinal fasciculus and impaired object recognition in children: a diffusion tensor imaging study. In: *Developmental Medicine & Child Neurology* 54 (1), S. 38–43. DOI: 10.1111/j.1469-8749.2011.04147.x.

Ouyang, X.; Tao, H. J.; Liu, H. H.; Deng, Q. J.; Sun, Z. H.; Xu, L. et al. (2011): White matter integrity deficit in treatment-naïve adult patients with major depressive disorder. In: *East Asian archives of psychiatry : official journal of the Hong Kong College of Psychiatrists = Dong Ya jing shen ke xue zhi : Xianggang jing shen ke yi xue yuan qi kan* 21 (1), S. 5–9.

Parras, Carlos M.; Hunt, Charles; Sugimori, Michiya; Nakafuku, Masato; Rowitch, David; Guillemot, François (2007): The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. In: *J. Neurosci.* 27 (16), S. 4233–4242. DOI: 10.1523/JNEUROSCI.0126-07.2007.

Petrides, Michael; Pandya, Deepak N. (2012): Chapter 26 - The Frontal Cortex. In: George Paxinos und Jürgen K. Mai (Hg.): The human nervous system. 3rd ed. Amsterdam, Boston: Elsevier Academic Press, S. 988–1011. Online verfügbar unter http://www.sciencedirect.com/science/article/pii/B9780123742360100264.

Rajkowska, Grazyna; Mahajan, Gouri; Maciag, Dorota; Sathyanesan, Monica; Iyo, Abiye H.; Moulana, Mohadetheh et al. (2015): Oligodendrocyte morphometry and expression of myelin - Related mRNA in ventral prefrontal white matter in major depressive disorder. In: *Journal of psychiatric research* 65, S. 53–62. DOI: 10.1016/j.jpsychires.2015.04.010.

Rogers, Mark A.; Bradshaw, John L.; Pantelis, Christos; Phillips, James G. (1998): Frontostriatal deficits in unipolar major depression. In: *Brain Research Bulletin* 47 (4), S. 297–310. DOI: 10.1016/S0361-9230(98)00126-9.

Ryu, Elizabeth J.; Wang, James Y. T.; Le, Nam; Baloh, Robert H.; Gustin, Jason A.; Schmidt, Robert E.; Milbrandt, Jeffrey (2007): Misexpression of Pou3f1 results in peripheral nerve hypomyelination and axonal loss. In: *J. Neurosci.* 27 (43), S. 11552–11559. DOI: 10.1523/JNEUROSCI.5497-06.2007.

Saher, Gesine; Brügger, Britta; Lappe-Siefke, Corinna; Möbius, Wiebke; Tozawa, Ryu-ichi; Wehr, Michael C. et al. (2005): High cholesterol level is essential for myelin membrane growth. In: *Nat Neurosci* 8 (4), S. 468–475. DOI: 10.1038/nn1426.

Schwartz, G. E.; Fair, P. L.; Mandel, M. R.; Salt, P.; Mieske, M.; Klerman, G. L. (1978): Facial electromyography in the assessment of improvement in depression. In: *Psychosomatic medicine* 40 (4), S. 355–360. DOI: 10.1097/00006842-197806000-00008.

Siegert, R. J.; Abernethy, D. A. (2005): Depression in multiple sclerosis: a review. In: *Journal of neurology, neurosurgery, and psychiatry* 76 (4), S. 469–475. DOI: 10.1136/jnnp.2004.054635.

Simpson, J. E.; Fernando, M. S.; Clark, L.; Ince, P. G.; Matthews, F.; Forster, G. et al. (2007): White matter lesions in an unselected cohort of the elderly: astrocytic, microglial and oligodendrocyte precursor cell responses. In: *Neuropathology and Applied Neurobiology* 33 (4), S. 410–419. DOI: 10.1111/j.1365-2990.2007.00828.x.

Srivastava, Shruti; Bhatia, Manjeet S.; Bhargava, S. K.; Kumari, Rima; Chandra, Sushil (2016): A Diffusion Tensor Imaging Study Using a Voxel-Based Analysis, Region-of-Interest Method to Analyze White Matter Abnormalities in First-Episode, Treatment-Naïve Major Depressive Disorder. In: *The Journal of neuropsychiatry and clinical neurosciences* 28 (2), S. 131–137. DOI: 10.1176/appi.neuropsych.15050120.

Staudt, M.; Krägeloh-Mann, I.; Grodd, W. (2000): Die normale Myelinisierung des kindlichen Gehirns in der MRT--eine Metaanalyse. In: *RoFo : Fortschritte auf dem Gebiete der Rontgenstrahlen und der Nuklearmedizin* 172 (10), S. 802–811. DOI: 10.1055/s-2000-7898.

Sugimoto, Koichiro; Kakeda, Shingo; Watanabe, Keita; Katsuki, Asuka; Ueda, Issei; Igata, Natsuki et al. (2018): Relationship between white matter integrity and serum inflammatory cytokine levels in drug-naive patients with major depressive disorder: diffusion tensor imaging study using tract-based spatial statistics. In: *Translational psychiatry* 8 (1), S. 141. DOI: 10.1038/s41398-018-0174-y.

Svebak, Sven (1988): Chapter 9 Psychogenic Muscle Tension. In: M. P. Cowles, M. J. Apter und J. H. Kerr (Hg.): Progress in Reversal Theory, Bd. 51. 1. Aufl. s.l.: Elsevier textbooks (Advances in Psychology, 51), S. 143–162.

Szebeni, Attila; Szebeni, Katalin; DiPeri, Timothy P.; Johnson, Luke A.; Stockmeier, Craig A.; Crawford, Jessica D. et al. (2017): Elevated DNA Oxidation and DNA Repair Enzyme Expression in Brain White Matter in Major Depressive Disorder. In: *The international journal of neuropsychopharmacology* 20 (5), S. 363–373. DOI: 10.1093/ijnp/pyw114.

Tanti, A.; Kim, J. J.; Wakid, M.; Davoli, M-A; Turecki, G.; Mechawar, N. (2018): Child abuse associates with an imbalance of oligodendrocyte-lineage cells in ventromedial prefrontal white matter. In: *Molecular psychiatry* 23 (10), S. 2018–2028. DOI: 10.1038/mp.2017.231.

Tham, Ming Wei; Woon, Puay San; Sum, Min Yi; Lee, Tih-Shih; Sim, Kang (2011): White matter abnormalities in major depression: evidence from post-mortem, neuroimaging and genetic studies. In: *Journal of affective disorders* 132 (1-2), S. 26–36. DOI: 10.1016/j.jad.2010.09.013.

Torres-Platas, Susana G.; Hercher, Christa; Davoli, Maria Antonietta; Maussion, Gilles; Labonté, Benoit; Turecki, Gustavo; Mechawar, Naguib (2011): Astrocytic hypertrophy in anterior cingulate white matter of depressed suicides. In: *Neuropsychopharmacol* 36 (13), S. 2650–2658. DOI: 10.1038/npp.2011.154.

Trepel, Martin; Dalkowski, Katja (2017): Neuroanatomie. Struktur und Funktion. 7th ed. Philadelphia: Urban & Fischer Verlag GmbH & Co. KG.

Valencia-Sanchez, Marco Antonio; Liu, Jidong; Hannon, Gregory J.; Parker, Roy (2006): Control of translation and mRNA degradation by miRNAs and siRNAs. In: *Genes Dev.* 20 (5), S. 515–524. DOI: 10.1101/gad.1399806.

van der Marel, Kajo; Homberg, Judith R.; Otte, Willem M.; Dijkhuizen, Rick M. (2013): Functional and structural neural network characterization of serotonin transporter knockout rats. In: *PloS one* 8 (2), e57780. DOI: 10.1371/journal.pone.0057780.

Vecera, Shaun P.; Rizzo, Matthew (2003): Spatial attention: normal processes and their breakdown. In: *Neurologic clinics* 21 (3), S. 575–607. DOI: 10.1016/s0733-8619(02)00103-2.

Videbech, Poul; Ravnkilde, Barbara (2004): Hippocampal volume and depression: a meta-analysis of MRI studies. In: *The American journal of psychiatry* 161 (11), S. 1957–1966. DOI: 10.1176/appi.ajp.161.11.1957.

Wang, Fei; Kalmar, Jessica H.; Edmiston, Erin; Chepenik, Lara G.; Bhagwagar, Zubin; Spencer, Linda et al. (2008): Abnormal corpus callosum integrity in bipolar disorder: a diffusion tensor imaging study. In: *Biological Psychiatry* 64 (8), S. 730–733. DOI: 10.1016/j.biopsych.2008.06.001.

Wang, Junhui; Qiao, Jinping; Zhang, Yanbo; Wang, Hongxing; Zhu, Shenghua; Zhang, Handi et al. (2014): Desvenlafaxine prevents white matter injury and improves the decreased phosphorylation of the rate-limiting enzyme of cholesterol synthesis in a chronic mouse model of depression. In: *Journal of neurochemistry* 131 (2), S. 229–238. DOI: 10.1111/jnc.12792.

Won, Eunsoo; Kang, June; Choi, Sunyoung; Kim, Aram; Han, Kyu-Man; Yoon, Ho-Kyoung et al. (2017): The association between substance P and white matter integrity in medication-naive patients with major depressive disorder. In: *Scientific reports* 7 (1), S. 9707. DOI: 10.1038/s41598-017-10100-y.

Wu, Feng; Tang, Yanqing; Xu, Ke; Kong, Lingtao; Sun, Wenge; Wang, Fei et al. (2011): Whiter matter abnormalities in medication-naive subjects with a single short-duration episode of major depressive disorder. In: *Psychiatry research* 191 (1), S. 80–83. DOI: 10.1016/j.pscychresns.2010.09.002.

Wu, Yupeng; Sun, Dandan; Wang, Yong; Wang, Yibao; Ou, Shaowu (2016): Segmentation of the Cingulum Bundle in the Human Brain: A New Perspective Based on DSI Tractography and Fiber Dissection Study. In: *Frontiers in Neuroanatomy* 10, S. 84. DOI: 10.3389/fnana.2016.00084.

Xiao, Qian; Wang, Feifei; Luo, Yanmin; Chen, Linmu; Chao, Fenglei; Tan, Chuanxue et al. (2018): Exercise protects myelinated fibers of white matter in a rat model of depression. In: *The Journal of comparative neurology* 526 (3), S. 537–549. DOI: 10.1002/cne.24350.

Yang, Xin-Hua; Wang, Yi; Wang, Dong-Fang; Tian, Kai; Cheung, Eric F. C.; Xie, Guang-Rong; Chan, Raymond C. K. (2017): White matter microstructural abnormalities and their association with anticipatory anhedonia in depression. In: *Psychiatry research. Neuroimaging* 264, S. 29–34. DOI: 10.1016/j.pscychresns.2017.04.005.

Yuan, Kai; Qin, Wei; Liu, Peng; Zhao, Ling; Yu, Dahua; Zhao, Limei et al. (2012): Reduced fractional anisotropy of corpus callosum modulates inter-hemispheric resting state functional connectivity in migraine patients without aura. In: *PloS one* 7 (9), e45476. DOI: 10.1371/journal.pone.0045476.

Zalsman, Gil; Weller, Aron; Shbiro, Liat; Barzilay, Ran; Gutman, Avihay; Weizman, Abraham et al. (2017): Fibre tract analysis using diffusion tensor imaging reveals aberrant connectivity in a rat model of depression. In: *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry* 18 (8), S. 615–623. DOI: 10.1080/15622975.2016.1190866.

Zhou, Yan; Qin, Ling-di; Chen, Jun; Qian, Li-jun; Tao, Jing; Fang, Yi-ru; Xu, Jian-rong (2011): Brain microstructural abnormalities revealed by diffusion tensor images in patients with treatment-resistant depression compared with major depressive disorder before treatment. In: *European journal of radiology* 80 (2), S. 450–454. DOI: 10.1016/j.ejrad.2010.06.041.

Zhu, Xueling; Wang, Xiang; Xiao, Jing; Zhong, Mingtian; Liao, Jian; Yao, Shuqiao (2011): Altered white matter integrity in first-episode, treatment-naive young adults with major

depressive disorder: a tract-based spatial statistics study. In: *Brain research* 1369, S. 223–229. DOI: 10.1016/j.brainres.2010.10.104.

Zonta, Barbara; Tait, Steven; Melrose, Shona; Anderson, Heather; Harroch, Sheila; Higginson, Jennifer et al. (2008): Glial and neuronal isoforms of Neurofascin have distinct roles in the assembly of nodes of Ranvier in the central nervous system. In: *The Journal of cell biology* 181 (7), S. 1169–1177. DOI: 10.1083/jcb.200712154.

Chapter 3 |
Consequences of early life stress

Early life stress and DNA methylation

Annakarina Mundorf [1] and Nadja Freund [1]

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

In: The DNA, RNA, and Histone Methylomes., RNA Technologies. Springer International Publishing. (2019) Book chapter, published

Early Life Stress and DNA Methylation

Annakarina Mundorf and Nadja Freund

Contents

Abstract DNA methylation and demethylation can be influenced by several environmental factors including diet, smoking, drug consumption, parental behavior and stress. Given that methylation changes can lead to altered gene transcription their impact can be enormous. Therefore, it is very important to understand the processes and underlying factors influencing methylation. Changes in DNA methylation that occur early during development induce altered gene expression that can affect the development of the brain and other organs right from the beginning. Stress during early development is linked to an increased risk for psychiatric and physiological disorders and altered DNA methylation could be the mediating factor. Whether the addition or the removal of methyl groups is linked to psychiatric outcome depends on several factors like the specific gene and the exposure. There are different approaches to investigate this relationship and to identify risk genes. Some groups focus on the mediating effect of gene methylation on early life stress exposure and psychiatric outcome. Another approach is the study of gene methylation in adults

A. Mundorf · N. Freund (✉)
Research Division Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Bochum, Germany
e-mail: nadja.freund@rub.de

S. Jurga, J. Barciszewski (eds.), *The DNA, RNA, and Histone Methylomes*, RNA Technologies, https://doi.org/10.1007/978-3-030-14792-1_9

with already diagnosed psychiatric disorders. Others investigated the reversible effect of psychotherapy on gene methylation in patients. Only a few studies correlate gene methylation in healthy adults with subclinical symptoms.

The following chapter will first give a brief introduction on environmental influences, DNA methylation and increased risk for the development of psychiatric disorders. It will then summarize findings in human and animal studies on early life stress, gene methylation and stress-related psychiatric disorders. At the end of the chapter, we will give an outlook on possible clinical applications.

Keywords Psychopathology · Lifestyle · Glucocorticoid receptor · Animal models · Maternal separation · Gene expression · Behavior

1 Environmental Influences on DNA Methylation

DNA methylation is either the spontaneous alteration of gene expression or the reaction to environmental influences, a post-replication modification by adding or removing a methyl group at a CpG side (Jaenisch and Bird 2003). It often occurs during the development of the organism and therefore changes are carried on through mitosis. In general, differences in gene expression are a mechanism that allows heterogeneity between genetically homologous cells. Environmental factors are known to influence DNA methylation patterns, thereby altering gene function and expression (Razin and Riggs 1980; Razin and Cedar 1991) and even causing disorders. Epigenetic modifications have been associated with various diseases like cancer, cardiovascular and neurodegenerative diseases as well as psychiatric disorders (Santos-Rebouças and Pimentel 2007; Mahgoub and Monteggia 2013). Therefore, understanding epigenetic processes and influences on the organism is an important step in clinical research. In cancer research the field of cancer epigenetics is rapidly growing and epigenetic therapy is making promising progress (Sharma et al. 2010). Driven by the success in cancer, other fields including psychiatry are now starting to also investigate epigenetics involved in disease processes.

1.1 Lifestyle

Not only severe life events but several environmental factors can influence DNA methylation. An individual's lifestyle, physical activities, nutrition, alcohol consumption, smoking or stress are known to induce DNA methylation changes (Alegría-Torres et al. 2011; Lim and Song 2012). Physical activity e.g. has been associated with higher methylation of the *LINE-1* gene which is linked to a reduced risk of ischemic heart disease and stroke in elderly (Alegría-Torres et al. 2011).

The abuse of substances including cocaine, opioids or alcohol is well-known for altering the DNA methylation state of specific genes (Nielsen et al. 2012). Altered

gene expression that arises as consequence might then be responsible for dysfunction in reward signaling, craving, and relapse leading to addiction and relapse (Nielsen et al. 2012). Even recent alcohol intake (i.e. consumption in the past 6 month) is known to change gene methylation in healthy participants (investigated in blood cells), leading to altered gene expression (Philibert et al. 2012; Liu et al. 2018). Thereby methylation changes depend on drinking frequency and indicate that higher amounts of consumed alcohol might lead to stronger DNA methylation changes (Philibert et al. 2012). Smoking can also effect DNA methylation in a dose- and time-dependent manner (Philibert et al. 2014). Altered DNA methylation induced by smoking affects gene regulation and is hypothesized to increase vulnerability to other diseases (Philibert et al. 2014).

Not only the amount but also timing of exposure or consumption is crucial. Vulnerable windows for environmental influences are especially periods of neural growth like early childhood or adolescence (Andersen and Teicher 2008; Freund et al. 2013). Therefore, lifestyle of a pregnant woman can even have long term consequences for the unborn child. DNA methylation changes induced by prenatal exposure might alter gene expression patterns even before crucial brain structures or pathways are fully developed (Lucassen et al. 2013) and induce long-lasting DNA methylation changes which e.g. increase the risk to develop psychiatric disorders later in life (Knopik et al. 2019). An unhealthy diet during pregnancy e.g. can affect DNA methylation of insulin-like growth factor 2 gene (*IGF2*) of the offspring and increase the risk to develop attention-deficit/hyperactivity disorder (ADHD) symptoms (Rijlaarsdam et al. 2017). Perinatal malnutrition also affects hippocampal growth via altered DNA methylation and increase the risk to develop psychiatric disorders (Lucassen et al. 2013).

1.2 Stress

Stress is known to affect an individual at many different levels. Influences on the immune and cardiovascular system as well as the brain can induce various diseases (McEwen and Stellar 1993; Romeo 2016). Specifically, during development, like early childhood and adolescence, the brain is vulnerable to stress exposure resulting in an increased risk to develop mental disorders consequently. Severe stress exposure is linked to several psychiatric disorders including major depressive disorder (MDD), bipolar disorder, posttraumatic stress disorder (PTSD) and schizophrenia (Vinkers et al. 2015; Matosin et al. 2017). Changes induced by stress exposure leading to the onset of a psychiatric disorder might be mediated by epigenetic alteration (Klengel et al. 2014; Vinkers et al. 2015; Matosin et al. 2017; Barker et al. 2017). Thereby, timing of stress exposure is critical as mentioned above. Consequences on the individual's development and health depend if stress exposure occurred prenatally, in early childhood or in adolescence (Matosin et al. 2017).

A study investigating DNA methylation changes (in buccal cells) in 15-year-old adolescents reported altered methylation after experiencing a stressful childhood (Essex et al. 2013). Interestingly, they found different time windows for maternal and paternal stress impacting the child's DNA methylation. Maternal stressors in infancy and paternal stressors in preschool years (parental stressors being e.g. financial stress, parenting stress, depression) were predictive for several DNA methylation changes (Essex et al. 2013). Methylation changes following stress exposure have been reported on several genes. The glucocorticoid receptor gene (*NR3C1*), the serotonin transporter gene (*SLC6A4*) and *FKBP5* (a gene involved in the immune system and interaction with the glucocorticoid receptor), however, seem to be mainly affected (Vinkers et al. 2015).

2 Findings in Human and Animal Studies Regarding Early Life Stress, Gene Methylation, and Psychiatric Disorders

2.1 Human Studies

Early life stress (ELS) affects DNA methylation patterns (Vinkers et al. 2015) and ample evidence indicates that ELS increases the risk for several psychiatric disorders (Teicher et al. 2003). Therefore, it is of special interest to link the reported methylation changes after ELS to psychopathology. Indeed methylation of the serotonin transporter gene *SLC6A4* has been reported to be altered in patients with bipolar disorder, MDD, PTSD, schizophrenia, ADHD and obesity (Palma-Gudiel and Fañanás 2017). Similarly, MDD and PTSD have been linked to methylation changes of the genes *NR3C* and *FKBP5* while increased methylation of *NR3C1* changes have also been reported in borderline personality disorder and patients with bipolar disorder show increased methylation on *FKBP5* (Argentieri et al. 2017).

Identifying methylation changes that mediate psychopathology after ELS, however, entails some challenges. Different aspects of life like smoking, nutrition, and parental behavior can influence DNA methylation. Therefore, it is hard to identify if altered DNA methylation is caused by a specific ELS and not being influenced by other environmental factors. Cause and consequence of methylation changes and psychopathology are hard to identify. Nevertheless, first studies try to identify the direct link between ELS, methylation and psychopathology. Specifically for the glucocorticoid receptor, there are some hints that this link exists (Smart et al. 2015). Radtke and colleagues e.g. assessed the occurrence of lifetime childhood maltreatment in 46 individuals, measured the methylation of the glucocorticoid receptor in blood samples and conducted a structured interview to evaluate psychological wellbeing. They found that number of events of maltreatment correlated positively with methylation and symptoms of borderline personality disorder and depression also positively correlated with methylation (Radtke et al. 2015). Longitudinal,

population-based studies also support the role of DNA methylation in mediating psychopathology after ELS (Barker et al. 2017). One example is the Avon Longitudinal Study of Parents and Children (ALSPAC) which is an ongoing epidemiological study of children and parents gathering psychological and physiological information e.g. whole-genome methylation data at different time points (Boyd et al. 2013; Fraser et al. 2013). With an integrated data resource for epigenomic studies (called ARIES) longitudinal, population-based DNA methylation profiling with a great number of subjects (N = 1018) is possible (Relton et al. 2015). Out of this complex study, multiple smaller studies have been conducted investigating the role of DNA methylation in connecting ELS and psychopathology. It has been found that methylation of the oxytocin receptor gene (*OXTR*) is associated with higher prenatal risk exposure and with higher callous-unemotional traits in adolescence (Cecil et al. 2014). *OXTR* methylation could therefore mediate callous-unemotional traits, a risk factor for developing early-onset conduct problems (CP).

In another epigenome-wide analysis, cord blood DNA methylation of children from the ALSPAC has been examined to investigate if altered DNA methylation could serve as a biomarker to detect early-onset CP (Cecil et al. 2018). Specifically, they used trajectories of CP to search for DNA methylation alterations between early-onset versus low CP and analyzed if early exposure influences DNA methylation. For example, maternal smoking showed a strong correlation with increased DNA methylation of *MGLL*, a gene encoding for a protein involved in pain perception, and was associated with early-onset CP in late childhood (Cecil et al. 2018).

Apart from the timing of stress exposure (prenatal, early life, adolescence) most epigenome-wide studies differ in (*I*) investigated cell types (umbilical cord blood, leukocytes, neonatal cord blood, blood, buccal cells, serum, brain tissue of suicide cases), (*II*) trauma type being chosen as ELS (childhood sexual or physical abuse, neglect, maltreatment, parental death/loss, institutionalized children), (*III*) technique being used for methylation analysis and (*IV*) the way of analyzing the results (genome-wide or candidate gene approach) (Vinkers et al. 2015). This high variability in the study design complicates validation of results and therewith, might weaken the potential role of DNA methylation as a mediator or biomarker for psychopathologies (Barker et al. 2017).

Another obstacle in finding methylation markers that link ELS and psychopathology is that ELS affects the brain and leads to neuroanatomical changes like volume reduction, of e.g. the hippocampus, the amygdala, the prefrontal cortex, and the corpus callosum (Hart and Rubia 2012; Frodl and O'Keane 2013). Changes in DNA methylation in peripheral tissue, however, do not reflect on the brain as DNA methylation is tissue specific (Bakulski et al. 2016). Studies in human post-mortem brain tissue are rare and limited (Vinkers et al. 2015). Animal models might help to overcome this obstacle. They are an excellent tool to investigate early life experience in a very controlled manner and link its effect on methylation changes in brain tissue to behavioural consequences.

2.2 Animal Studies

A well-established animal model for ELS is maternal separation (MS) (Freund et al. 2013; Nieto et al. 2016). Briefly, the pups are separated from their mother for several hours each day during their early childhood. Affected brain regions and behavioral phenotype following MS are comparable to findings in humans who experienced ELS (Teicher et al. 2003). Following MS, multiple DNA methylation changes in genes (frequently relevant in brain maturation or plasticity) in different brain regions (e.g. hippocampus, hypothalamus, amygdala, prefrontal cortex) have been found (Fumagalli et al. 2007; Nieto et al. 2016). Regarding the psychopathology, conclusions can be drawn by investigating the behavior of the animal. As well as in humans, disorders are first characterized by alterations in the normal behavior. Therewith, increased anxiety, depressive-like, hedonic or anhedonic behavior can be measured as well as impaired memory, stress coping and impulsiveness. Standardized assays have been developed and validated to investigate psychiatric psychopathologies in rodents (Nestler and Hyman 2010). For example, by using the natural aversion of rodents towards open spaces, altered anxiety behavior can be assessed by exposing them to a large open field (open field test).

Investigating effects of ELS on DNA methylation, an increased methylation of *Nr3c1* as shown in humans has also been confirmed in the hippocampus of maternally separated mice (Kember et al. 2012). These animals furthermore showed increased methylation of the gene for vasopressin *Avp* and decreased methylation of *Nr4a1*, a growth factor gen (Kember et al. 2012). Changes in methylation status were furthermore accompanied by increased anxiety behavior and an increased corticosterone response to stress (Kember et al. 2012). In the hypothalamus MS resulted in decreased methylation of the corticotrophin gen *Crh* (Chen et al. 2012) and *Avp* (Murgatroyd et al. 2009). Another benefit of animal studies is the analysis of DNA methylation and mRNA expression at once. The decreased methylation of *Avp* resulted in increased vasopressin expression and animals showing alterations in stress coping and memory. Interestingly, a vasopressin receptor agonist was able to reverse the behavioral changes (Murgatroyd et al. 2009) reinforcing the important role of *Avp* in stress coping and memory behavior. Synapsin gen *Syn1* was more methylated in the amygdala after MS resulting in lower mRNA expression (Park et al. 2014). In the same region increased methylation of *Ntsr1* (encoding for the neurotensin reporter 1) induced enhanced fear conditioning and reduced gene expression of *Ntsr1* (Toda et al. 2014). Microinjection of a neurotensin receptor agonist or antagonist was able to de- or increase fear conditioning, respectively (Toda et al. 2014). All studies reinforce the mediating role of methylation. In the pituitary gland, MS caused decreased methylation of the Proopiomelanocortin gen *Pomc* (a gene involved in stress and immune modulation) that was accompanied by an increase in mRNA expression (Wu et al. 2014). Furthermore, the reduction of reward seeking after MS was accompanied by hypermethylation of the Dopamine D1 receptor in the nucleus accumbens and a consequent decrease of mRNA as well as protein levels (Sasagawa et al. 2017). These studies confirm the potential role of

DNA methylation in mediating the development of psychopathologies after experiencing ELS.

First hints for treatment options were shown in animal models as well. Adding a methyl donor to the animals diet improved anxiety and depressive-like behavior after MS (McCoy et al. 2016; Paternain et al. 2016). As the methyl donor enriched diet was given when the MS animals were adults the behavioral alterations induced by MS are still reversible later on. The preventive effects of an enriched diet has been suggested in humans as well (Rijlaarsdam et al. 2017).

But not only separation from the mother, even low maternal care can influence DNA methylation. Disrupted maternal care increased the expression of the DNA methyltransferase in the offspring's amygdala leading to an increase in total methylation and increased anxiety. Abusive maternal behavior increased methylation of the *Bdnf* gene, coding for a neurotrohic factor, as well as decreased its mRNA expression in the prefrontal cortex (Roth et al. 2009). This fact is in line with results found in human patients with borderline personality disorder showing increased *BDNF* methylation (Thomas et al. 2018, see Sect. 3). In the animal model, a potential treatment option was already discovered. Infusing the grown up rats with a DNA methylation inhibitor resulted in decreased methylation of the *Bdnf* gene and normalized mRNA expression (Roth et al. 2009). Increased maternal care, on the other hand, can have beneficial effects. Increased licking and grooming of the pups decreased the methylation of the glucocorticoid gene in the offspring's hippocampus (Weaver et al. 2004). This alteration was persistent into adulthood and might influence the stress response via the hypothalamic-pituitary-adrenal axis. So, early maternal care might have a high effect on the stress response of the offspring by affecting brain plasticity.

The findings in animal studies emphasize the relevance of DNA methylation changes that disrupt brain maturation, especially, when the methylation changes are induced during vulnerable windows. Nevertheless, most studies only investigate the effects of ELS on DNA methylation without considering the consequences on psychopathology (behavior). In addition, apart from the glucocorticoid receptor, human studies point to different genes compared to animal studies. A recently conducted evaluation of human and animal studies on ELS and DNA methylation found that results match in less than 50% between animal models and studies with humans (Watamura and Roth 2018). One explanation for this finding could be that, preclinical (animal) and clinical (human) studies are not well-aligned and often differ in time of stress exposure, type of stressor, timing of tissue sampling and tissue investigated (Nieto et al. 2016). Cross-species, multi-tissue studies to investigate the effect of ELS on DNA methylation help to identify stable effects that are comparable across species.

2.3 Cross Species Studies

Nieratschker and colleagues compared DNA methylation of the *MORC family CW-type zinc finger 1* (*MORC1*) gene from (*I*) human cord blood following prenatal stress, (*II*) the prefrontal cortex tissue of adult rats that had been exposed to prenatal

stress, and (*III*) blood cells of adolescent nonhuman primates after maternal separation with (*IV*) matched non-stressed control groups (Nieratschker et al. 2014). They found reduced methylation of *MORC1* in all tissues of all species. Then, they went one step further and performed a gene-based case-control analysis with data from a previous genome-wide association study with blood from major depression patients. Interestingly, specific gene variants of *MORC1* were associated with major depressive disorder (Nieratschker et al. 2014).

Similarly, methylation profiles in post-mortem hippocampus samples of suicide victims with and without a history of severe childhood abuse were studied (Suderman et al. 2012). The findings were then compared with results in hippocampus samples of rats that received low or high maternal care. Interestingly, they found high similarities of methylation of the glucocorticoid receptor gene after ELS in humans and rats indicating cross-species regulatory mechanisms that are conserved. As mentioned, altered methylation of the glucocorticoid receptor gene after ELS has already been reported in human blood (Radtke et al. 2015; Smart et al. 2015) and rat hippocampus (Weaver et al. 2004). So far, this alteration has been linked with increased risk of MDD and stress related disorders like PTSD. Therewith, it seems possible that methylation of the glucocorticoid receptor gene might rather play a role in stress reaction than in the development of a specific disorder. Even though cross species studies hold promise there are still rare and more studies are urgently needed.

3 Possible Clinical Applications of Gene Methylation: An Outlook

As stated in the beginning, there are different approaches to investigate the effects of ELS and DNA methylation on psychopathology. We reported that DNA methylation changes have been found when comparing humans and animals with or without stress experience or between patients and healthy controls.

Another approach is to correlate symptoms and methylation patterns in population wide studies. In a recent study with healthy adults (30 women and 30 men), we found a significant association between increased *MORC1* promoter methylation in buccal cells and increased self-depression scoring according to the Beck Depression Inventory (Mundorf et al. 2018). Increased *MORC1* methylation could potentially serve as an early detection marker for depressive symptoms. Similarly, methylation patterns of several genes might act as biomarker for early detection of psychiatric disorders. One advantage of early detection is that our epigenetic pattern including DNA methylation is changeable. As mentioned above, drugs can influence our DNA methylation signature. Therefore it is no surprise, that pharmacological treatment alters DNA methylation (Boks et al. 2012; Ovenden et al. 2018). But even psychotherapy can alter DNA methylation. The serotonin transporter as well as *FKBP5* showed changes in methylation pattern after psychotherapy (Roberts et al. 2014, 2019). Even more interesting, stronger changes in methylation pattern are associated

with a greater response to the therapy. Therefore, DNA methylation could potentially serve as marker for therapy outcome.

A different but interesting approach is the study of gene methylation in adults with already diagnosed psychiatric disorders. Especially, as early stress exposure is linked to an increased risk of a psychiatric disorder and altered DNA methylation. Identifying risk genes in patients will facilitate early diagnosis.

In a recent clinical study in borderline personality disorder patients (85% women) and matched controls, *BDNF* promoter methylation was investigated in buccal and blood cells (Thomas et al. 2018). Interestingly, the researchers found a significantly increased *BDNF* promoter methylation in the patients but only in saliva samples. Moreover, they investigated the effects of psychotherapy on *BDNF* methylation in the patients which decreased significantly after 12-month treatment (Thomas et al. 2018). More than half of the borderline patients declared having experienced childhood traumata. These findings seems to be in line with previous findings in animal studies where an increased methylation of *Bdnf* in the prefrontal cortex was found after experiencing abusive maternal care (Roth et al. 2009). In the animal study, treating the grown-up rats with a DNA methylation inhibitor resulted in decreased methylation of the *Bdnf* gene and normalized mRNA expression giving hope for new and effective treatment options of borderline personality disorder.

As mentioned above (see Sect. 1.1) alcohol consumption can alter DNA methylation. Whether alcohol consumption induces DNA methylation changes leading to addiction or DNA methylation changes leading to more consumption and therewith addictive behavior is unknown. For a better understanding, both scenarios have to be analyzed. In a study with alcohol-dependent patients (49 male patients; mean age 49.14 ± 10.47 years), the patients showed decreased methylation of *GDAP1*, a gene, that has so far only been associated with the neurological disorder Charcot-Marie-Tooth disease. Furthermore the analyses conducted in blood cells revealed that the degree of hypomethylation was associated with increased alcohol dependence (Brückmann et al. 2016). Methylation of *GDAP1* might therefore serve as a biomarker for disease severity. In addition, the comparison of *GDAP1* methylation before and after 3 weeks of an alcohol treatment program showed an increase of methylation after treatment (Brückmann et al. 2016). Other studies confirmed, that an altered DNA methylation signature in blood cells could enable clinical diagnosis of heavy alcohol consumption which is difficult to access in patients (Liu et al. 2018). As heavy alcohol consumption (and dependence) is frequent in patients with psychiatric disorders knowing the DNA methylation signature will facilitate differential diagnosis by DNA methylation.

Even though the recent findings in DNA methylation predicting psychopathology or treatment outcome seems promising, the application of DNA methylation as biomarker posses challenges. Most studies use case-control or cross-sectional methods and only a few apply a prospective approach. Therefore, it is still unclear whether gene methylation is a predisposing factor for diseases or a consequence of pathology (Argentieri et al. 2017).

4 Conclusions

In conclusion, human and animal studies report an effect of ELS on DNA methylation. In addition, ELS and altered DNA methylation have been linked to different psychiatric disorders. Therefore, altered DNA methylation might be the missing link between stress exposure and the development of psychopathology. So far, most studies use different designs or investigate different genes, making it difficult to validate results. Moreover, animal and human studies are not well aligned, and more cross-species studies are needed. Nevertheless, DNA methylation has the potential for an easy-to-apply biomarker not only to facilite diagnosis but also as an early detection marker of symptoms. Findings like the reversal effect of psychotherapy on DNA methylation or the successful treatment with a methyl donor put DNA methylation in the position of a possible clinical treatment target.

References

Alegría-Torres JA, Baccarelli A, Bollati V (2011) Epigenetics and lifestyle. Epigenomics 3:267–277

Andersen SL, Teicher MH (2008) Stress, sensitive periods and maturational events in adolescent depression. Trends Neurosci 31:183–191

Argentieri MA, Nagarajan S, Seddighzadeh B et al (2017) Epigenetic pathways in human disease: the impact of DNA methylation on stress-related pathogenesis and current challenges in biomarker development. EBioMedicine 18:327–350

Bakulski KM, Halladay A, Hu VW et al (2016) Epigenetic research in neuropsychiatric disorders: the "tissue issue". Curr Behav Neurosci Rep 3:264–274

Barker ED, Walton E, Cecil CAM (2017) Annual Research Review: DNA methylation as a mediator in the association between risk exposure and child and adolescent psychopathology. J Child Psychol Psychiatry 59:303–322

Boks MP, de Jong NM, Kas MJH et al (2012) Current status and future prospects for epigenetic psychopharmacology. Epigenetics 7:20–28

Boyd A, Golding J, Macleod J et al (2013) Cohort profile: The 'Children of the 90s'—the index offspring of the Avon Longitudinal Study of Parents and Children. Int J Epidemiol 42:111–127

Brückmann C, Di Santo A, Karle KN et al (2016) Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. Epigenetics 11:456–463

Cecil CAM, Lysenko LJ, Jaffee SR et al (2014) Environmental risk, oxytocin receptor gene (OXTR) methylation and youth callous-unemotional traits: a 13-year longitudinal study. Mol Psychiatry 19:1071–1077

Cecil CAM, Walton E, Jaffee SR et al (2018) Neonatal DNA methylation and early-onset conduct problems: a genome-wide, prospective study. Dev Psychopathol 30:383–397

Chen J, Evans AN, Liu Y et al (2012) Maternal deprivation in rats is associated with corticotrophin-releasing hormone (CRH) promoter hypomethylation and enhances CRH transcriptional responses to stress in adulthood. J Neuroendocrinol 24:1055–1064

Essex MJ, Boyce WT, Hertzman C et al (2013) Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. Child Dev 84:58–75

Fraser A, Macdonald-Wallis C, Tilling K et al (2013) Cohort profile: the Avon Longitudinal Study of parents and children: ALSPAC mothers cohort. Int J Epidemiol 42:97–110

Freund N, Thompson BS, Denormandie J et al (2013) Windows of vulnerability: maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. Neuroscience 249:88–97

Frodl T, O'Keane V (2013) How does the brain deal with cumulative stress? A review with focus on developmental stress, HPA axis function and hippocampal structure in humans. Neurobiol Dis 52:24–37

Fumagalli F, Molteni R, Racagni G et al (2007) Stress during development: Impact on neuroplasticity and relevance to psychopathology. Prog Neurobiol 81:197–217

Hart H, Rubia K (2012) Neuroimaging of child abuse: a critical review. Front Hum Neurosci 6:52

Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33(Suppl):245–254

Kember RL, Dempster EL, Lee THA et al (2012) Maternal separation is associated with strain-specific responses to stress and epigenetic alterations to Nr3c1, Avp, and Nr4a1 in mouse. Brain Behav 2:455–467

Klengel T, Pape J, Binder EB et al (2014) The role of DNA methylation in stress-related psychiatric disorders. Neuropharmacology 80:115–132

Knopik VS, Marceau K, Bidwell LC et al (2019) Prenatal substance exposure and offspring development: Does DNA methylation play a role? Neurotoxicol Teratol 71:50–63. https://doi.org/10.1016/j.ntt.2018.01.009

Lim U, Song M-A (2012) Dietary and lifestyle factors of DNA methylation. Methods Mol Biol Clifton NJ 863:359–376

Liu C, Marioni RE, Hedman ÅK et al (2018) A DNA methylation biomarker of alcohol consumption. Mol Psychiatry 23:422–433

Lucassen PJ, Naninck EFG, van Goudoever JB et al (2013) Perinatal programming of adult hippocampal structure and function; emerging roles of stress, nutrition and epigenetics. Trends Neurosci 36:621–631

Mahgoub M, Monteggia LM (2013) Epigenetics and psychiatry. Neurother J Am Soc Exp Neurother 10:734–741

Matosin N, Cruceanu C, Binder EB (2017) Preclinical and clinical evidence of DNA methylation changes in response to trauma and chronic stress. Chronic Stress Thousand Oaks Calif 1. https://doi.org/10.1177/2470547017710764

McCoy CR, Rana S, Stringfellow SA et al (2016) Neonatal maternal separation stress elicits lasting DNA methylation changes in the hippocampus of stress-reactive Wistar Kyoto rats. Eur J Neurosci 44:2829–2845

McEwen BS, Stellar E (1993) Stress and the individual. Mechanisms leading to disease. Arch Intern Med 153:2093–2101

Mundorf A, Schmitz J, Güntürkün O et al (2018) Methylation of MORC1: a possible biomarker for depression? J Psychiatr Res 103:208–211

Murgatroyd C, Patchev AV, Wu Y et al (2009) Dynamic DNA methylation programs persistent adverse effects of early-life stress. Nat Neurosci 12:1559–1566

Nestler EJ, Hyman SE (2010) Animal models of neuropsychiatric disorders. Nat Neurosci 13:1161–1169

Nielsen DA, Utrankar A, Reyes JA et al (2012) Epigenetics of drug abuse: predisposition or response. Pharmacogenomics 13:1149–1160

Nieratschker V, Massart R, Gilles M et al (2014) MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Transl Psychiatry 4:e429

Nieto SJ, Patriquin MA, Nielsen DA, Kosten TA (2016) Don't worry; be informed about the epigenetics of anxiety. Pharmacol Biochem Behav 146–147:60–72

Ovenden ES, McGregor NW, Emsley RA et al (2018) DNA methylation and antipsychotic treatment mechanisms in schizophrenia: progress and future directions. Prog Neuro-Psychopharmacol Biol Psychiatry 81:38–49

Palma-Gudiel H, Fañanás L (2017) An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR. Neurosci Biobehav Rev 72:190–209

Park H-J, Kim S-K, Kang W-S et al (2014) Increased activation of synapsin 1 and mitogen-activated protein kinases/extracellular signal-regulated kinase in the amygdala of maternal separation rats. CNS Neurosci Ther 20:172–181

Paternain L, Martisova E, Campión J et al (2016) Methyl donor supplementation in rats reverses the deleterious effect of maternal separation on depression-like behaviour. Behav Brain Res 299:51–58

Philibert RA, Plume JM, Gibbons FX et al (2012) The impact of recent alcohol use on genome wide DNA methylation signatures. Front Genet 3:54

Philibert RA, Beach SRH, Brody GH (2014) The DNA methylation signature of smoking: an archetype for the identification of biomarkers for behavioral illness. Neb Symp Motiv 61:109–127

Radtke KM, Schauer M, Gunter HM et al (2015) Epigenetic modifications of the glucocorticoid receptor gene are associated with the vulnerability to psychopathology in childhood maltreatment. Transl Psychiatry 5:e571

Razin A, Cedar H (1991) DNA methylation and gene expression. Microbiol Rev 55:451–458

Razin A, Riggs AD (1980) DNA methylation and gene function. Science 210:604–610

Relton CL, Gaunt T, McArdle W et al (2015) Data resource profile: accessible resource for integrated epigenomic studies (ARIES). Int J Epidemiol 44:1181–1190

Rijlaarsdam J, Cecil CAM, Walton E et al (2017) Prenatal unhealthy diet, insulin-like growth factor 2 gene (IGF2) methylation, and attention deficit hyperactivity disorder symptoms in youth with early-onset conduct problems. J Child Psychol Psychiatry 58:19–27

Roberts S, Lester KJ, Hudson JL, Rapee RM, Creswell C, Cooper PJ, Thirlwall KJ, Coleman JRI, Breen G, Wong CCY, Eley TC (2014) Serotonin tranporter methylation and response to cognitive behaviour therapy in children with anxiety disorders. Transl Psychiatry 4(9):e444

Roberts S, Keers R, Breen G, Coleman JRI, Jöhren P, Kepa A, Lester KJ, Margraf J, Scheider S, Teismann T, Wannemüller A, Eley TC, Wong CCY (2019) DNA methylation of FKBP5 and response to exposure-based psychological therapy. Am J Med Genet B Neuropsychiatr Genet 180(2):150–158

Romeo RD (2016) The impact of stress on the structure of the adolescent brain: implications for adolescent mental health. Brain Res 1654:185–191

Roth TL, Lubin FD, Funk AJ, Sweatt JD (2009) Lasting epigenetic influence of early-life adversity on the BDNF gene. Biol Psychiatry 65:760–769

Santos-Rebouças CB, Pimentel MMG (2007) Implication of abnormal epigenetic patterns for human diseases. Eur J Hum Genet 15:10–17

Sasagawa T, Horii-Hayashi N, Okuda A et al (2017) Long-term effects of maternal separation coupled with social isolation on reward seeking and changes in dopamine D1 receptor expression in the nucleus accumbens via DNA methylation in mice. Neurosci Lett 641:33–39

Sharma S, Kelly TK, Jones PA (2010) Epigenetics in cancer. Carcinogenesis 31:27–36

Smart C, Strathdee G, Watson S et al (2015) Early life trauma, depression and the glucocorticoid receptor gene – an epigenetic perspective. Psychol Med 45:3393–3410

Suderman M, McGowan PO, Sasaki A et al (2012) Conserved epigenetic sensitivity to early life experience in the rat and human hippocampus. Proc Natl Acad Sci U S A 109(Suppl 2):17266–17272

Teicher MH, Andersen SL, Polcari A et al (2003) The neurobiological consequences of early stress and childhood maltreatment. Neurosci Biobehav Rev 27:33–44

Thomas M, Knoblich N, Wallisch A et al (2018) Increased BDNF methylation in saliva, but not blood, of patients with borderline personality disorder. Clin Epigenetics 10:109

Toda H, Boku S, Nakagawa S et al (2014) Maternal separation enhances conditioned fear and decreases the mRNA levels of the neurotensin receptor 1 gene with hypermethylation of this gene in the rat amygdala. PLoS One 9:e97421

Vinkers CH, Kalafateli AL, Rutten BPF et al (2015) Traumatic stress and human DNA methylation: a critical review. Epigenomics 7:593–608

Watamura SE, Roth TL (2018) Looking back and moving forward: evaluating and advancing translation from animal models to human studies of early life stress and DNA methylation. Dev Psychobiol. https://doi.org/10.1002/dev.21796

Weaver ICG, Cervoni N, Champagne FA et al (2004) Epigenetic programming by maternal behavior. Nat Neurosci 7:847–854

Wu Y, Patchev AV, Daniel G et al (2014) Early-life stress reduces DNA methylation of the Pomc gene in male mice. Endocrinology 155:1751–1762

Reduced ultrasonic vocalization in adolescent rats in a test for anxiety

Annakarina Mundorf [1]*, Ibrahim Bölükbas [1], Nadja Freund [1]

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

*Corresponding author

Research article / submitted

Abstract

The study of ultrasonic vocalization (USV) allows new insights when investigating the affective state of rodents. USV holds potential to e.g. record anxiety and might enable less invasive analysis. Few studies have so far focused on vocalization in adolescence even though this is a crucial time of vulnerability to develop affective disorders. These few studies reveal different communication patterns for adolescents in positive situations. Here, we provide insights into juvenile, adolescent and maternal USV during an aversive situation. Juvenile, adolescent, and maternal (post-weaning) rats were tested in the elevated plus maze test, a test for anxiety. Using a Batlogger M., calls were recorded during testing and then sorted into 50 kHz frequencies and 22 kHz frequencies. Interestingly, adolescents emitted significantly less calls at both, 22 and 50 kHz frequencies compared to juvenile or dams. Time spent in open arms, however, did not differ between adolescents and dams but was increased in juveniles indicating less anxiety. USV communication in adolescence might therefore have a different meaning as in juvenility or adulthood and further investigation is needed to reveal its connection with affective state in this sensitive age group.

Keywords: Elevated plus maze, dams, Batlogger, Sprague-Dawley, juveniles, adults, affective, non-invasive

1. Introduction

Recently, the investigation and use of ultrasonic vocalization (USV) in rodents has become of interest to a broad variety of studies ranging from developmental studies (Branchi et al., 2001), pharmacological use (Brudzynski, 2015) to a tool for predicting behavior. In general, USV has been mainly categorized based on frequency. So far, 50 kHz vocalization is associated with positive emotional states emitted in rewarding situations and social interaction and 22 kHz calls have been linked to negative emotional states such as a state of alarm, discomfort, or danger (Brudzynski, 2015; Knutson et al., 2002). Thus, the analysis of USV allows for investigating the affective state of rodents. Therefore, this area of research might hold potential in the study of affective disorders (Burgdorf et al., 2020).

Research on USV has investigated its potential for prediction mostly in the areas of vulnerability and stress resilience in adults. A study analyzing USV as a predictor of stress resilience in adult male rats that were exposed to a swim test found that USV-emitting rats seem to be more resilient to stress than non-emitting rats (Drugan et al., 2009). Studies investigating whether USV emission is a passive or active response to fear used conditioning with avoidable and nonavoidable shocks. They found that adult rats receiving more stress also emit more USV regardless of conditioning (Kikusui et al., 2003). Using the formalin test to induce pain in adult rats, researchers were also able to suppress pain associated vocalization when administering morphine (Oliveira and Barros, 2006).

In some animal models, it is difficult to differentiate whether the reaction is a response to pain, fear, or anxiety. But studies investigating the neurological and behavioral correlates of vocalization, as well as pharmacological studies, proofed that measuring USV is a valid way of investigating emotional state of rats (for review see (Sánchez, 2003)). In a study using tail shock for fear conditioning and intertrial cues to induce anxiety, researchers were able to link 22 kHz vocalization to anxiety and not fear (Jelen et al., 2003). Moreover, they administered anxiolytic drugs which themselves suppressed USV emission while an anxiogenic drug led to enhanced vocalization during intertrial intervals (Jelen et al., 2003). Furthermore, exposing adult rats to 22 kHz and 50 kHz playback USV of another rat leads to increased anxiety-like behavior and neuronal activation patterns related to anxiety (Demaestri et al., 2019; Sadananda et al., 2008). Therefore, the study of USV holds potential to investigate affective disorders.

Interestingly, USV seems to have a different role not only during early postnatal development (Branchi et al., 2001) but also for adolescent rats compared to adults. A team investigating adolescent and adult vocalization in rats found that adolescents might be more hesitant than adults in emitting 50 kHz calls when placed in a new cage with no other animal

present (Schwarting, 2018). Moreover, it has been shown, that vocalization during playful interaction has a minor role in adolescent rats compared to a more central role in adult rats (Kisko et al., 2015). Adolescence is a period of change as the neuronal growth of different regions, especially the prefrontal cortex and the amygdala undergo rapid growth (for review see (Lupien et al., 2009)). Moreover, in humans the amygdala- prefrontal circuity undergoes a shift from positive (in childhood) to negative connectivity in adolescence (Gee et al., 2013). Meaning a greater regulation of the amygdala by the prefrontal cortex with increasing brain maturation. This is important, as both regions are part of the human social brain (Blakemore, 2012). Thus it comes to no surprise, that the peak onset for anxiety disorders, especially social anxiety is during adolescence (Kessler et al., 2005). When investigating affective disorders like anxiety disorder, adolescence is, therefore, a crucial time to study.

However, most studies investigating adolescent vocalization have focussed on 50 kHz calls but none on aversive 22 kHz-emitting situations. To strengthen the field of USV analysis for studying affective disorders, which predominantly start in adolescence, more insight into adolescent vocalization in aversive situations is needed. A broader understanding of adolescent vocalization in anxiety-related situations would benefit the study of affective disorders in adolescent animal models. Thus, it would be of interest to investigate whether adolescent USV in anxiety-related situations differs from juvenile or adult USV. In this study, USV was therefore recorded for 5 minutes during an elevated plus maze test performed by (I) juvenile and (II) adolescent male and female as well as (III) maternal Sprague-Dawley rats. Therewith, anxiety behavior and USV could be analyzed and compared between the different developmental stages. The elevated plus maze is a widely used test to assess anxiety in rodents (Walf and Frye, 2007). Briefly, the animals are exposed to a maze consisting of two open and two closed arms. Benefiting of the normal aversion of rodents towards open spaces, the time spent in the open arms can be used to assess anxiety.

2. Methods

2.1 Animals

Eleven timed pregnant Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were single housed upon arrival (between gestational days 13-15). The day of birth was considered postnatal day (PND) 0. At PND2 pups were sexed and culled to 10 when necessary, if possible five male and five female pups. As the animals included in this study were part of another study investigating the consequences of maternal separation, some animals had experiences maternal separation and for statistical analysis, maternal separation was added

as factor. After weaning (PND21), animals were either group-housed with same-sex littermates or single housed. All animals were housed under standard conditions (22± 2°C room temperature, 55 ± 25 % humidity) and standard lighting (12h/12h) with free access to water and food. Experiments were conducted under the principles of Germany's Animal Welfare Act after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Northrhine-Westfalia).

2.2 Anxiety testing and USV recording

The EPM consists of four arms (50 cm x 10 cm), forming a maze 50 cm from the ground. Two of the four arms are open whereas two arms are closed to provide protection. The behavioral testing was performed during the dark (= active) phase under red light only. Animals were allowed to run freely for 5 min while being filmed using an HD Webcam (C920 Pro, Logitech) connected to a laptop. USV was recorded using a Batlogger M (Elekon AG, Lucerne, Switzerland) positioned above one of the open arms without disturbing the animals. The minimum frequency was set to 15 kHz, the maximum frequency to 155 kHz with Volume = 2 and automatic setup trigger. Recorded USVs were analyzed using BatExplorer with FFT size of 1024 and an overlap of 80% (Elekon AG, Lucerne, Switzerland). Counted USVs were sorted into 50-kHz frequency range (33 – 90 kHz) and into 22-kHz frequency range (15 – 32.9 kHz) (Brudzynski, 2015).

Animals were tested at three different ages: juvenile at PND21 (10 female + 10 male), adolescent at PND41 (14 female + 19 male) and maternal dams being >PND100 (N = 11). Time spent in the open and close arms was assessed manually by a rater blinded to condition. Moreover, animal movement was assessed to control whether the animal spent more time next to the microphone than on the other open arm. Both arms were visited equally.

2.3 Statistical analysis

A multivariate analysis of variance (MANOVA) was conducted with age and maternal separation as factors and number of calls for 22 kHz, for 50 kHz and time spent open as dependent variables. Two values (1 dam and 1 juvenile) for 22 kHz calls had to be excluded due to outlier status as they were marked as extreme values by percentile analysis. Bonferroni analysis for age was used for post-hoc correction.

3. Results

3.1 Anxiety-like behavior

Age had a significant effect on time spent open $F(2,60) = 26.642$; $p = 0.000$, on 22 kHz calls $F(2,60) = 11.992$; $p = 0.000$ and on 50 kHz calls $F(2,60) = 20.827$; $p = 0.000$. Maternal separation had neither an effect on time spent open $F(1,61) = 1.023$, $p = 0.316$, or on 22 kHz calls $F(1,61) = 0.009$, $p = 0.927$ or on 50 kHz calls $F(1,61) = 0.290$; $p = 0.593$ as well.

Post-hoc test for age revealed that juvenile rats spent significantly more time on the open arms ($M = 3.04$, $SD = 0.75$) than adolescent ($M = 1.78$, $SD = 0.56$), $p = 0.000$ and dams ($M = 1.83$, $SD = 0.43$), $p = 0.000$. Adolescent and adult rats spent statistically equal time on the open arms, $p = 1.00$. Moreover, juvenile rats emit more calls in 22 kHz frequencies ($M = 55.37$, $SD = 65.2$) than adolescent ($M = 0.36$, $SD = 1.27$), $p = 0.000$. But adolescent rats emit less calls than dams ($M = 38.5$, $SD = 62.34$), $p = 0.035$. Juvenile and adult rats emit a statistically equal number of calls, $p = 0.873$. Post-hoc test showed also that juvenile rats emit more 50 kHz calls ($M = 69.65$, $SD = 47.42$) than adolescent ($M = 1.7$, $SD = 2.75$), $p = 0.000$. Again, adolescent emit less 50 kHz calls than dams ($M = 48.82$, $SD = 62.34$), $p = 0.001$. Juvenile and adult rats do not statistically differ in number of 50 kHz calls, $p = 1.000$.

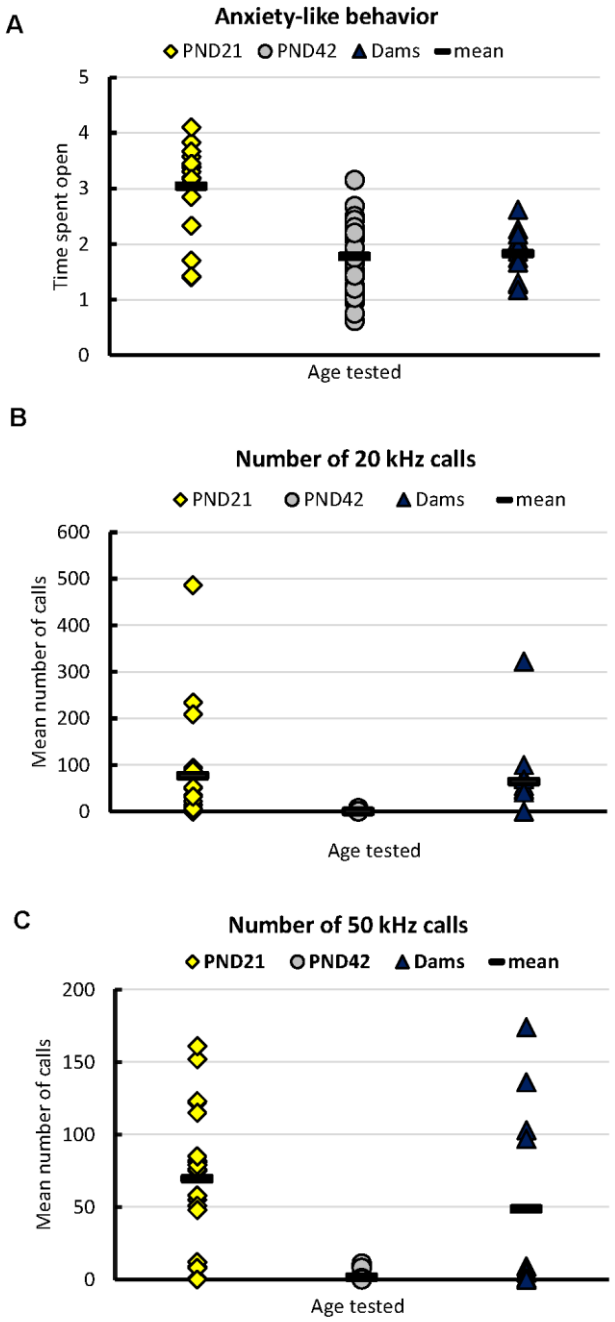


Figure 1: Anxiety behavior and ultrasonic vocalization. Data is presented as data points. The black bar represents the mean. **A**: Time spent in the open arms given in minutes. Juveniles spent more time in the open than adolescents or adults (p = .000). **B**: Adolescents emit significantly less calls at 22 kHz frequencies than juveniles (p = .000) or adults (p = .035).
C: Adolescents emit significantly less calls at 50 kHz frequencies than juveniles (p = .000) or adults (p = .001).

4. Discussion

In this study, adolescent vocalization during a test for anxiety was recorded and compared to juvenile and adult vocalization. Regarding anxiety, juvenile rats are less anxious in the EPM than adolescent or female adult rats. Adolescent and adult rats do not differ in anxiety.

Interestingly, adolescents emit generally significantly fewer calls during EPM testing whereas juvenile and maternal dams seem not to differ in the number of calls emitted.

The finding that juvenile rats were less anxious than adolescents or adults is in contrast to other studies reporting that juvenile rats are most anxious with decreasing anxiety over development until old adulthood (Lynn and Brown, 2010). Adolescence is usually connected to increased risk-taking and explorative behavior (Galván, 2010; Stansfield and Kirstein, 2006), therefore, less anxiety would be suspected in adolescence. One might argue that the apparatus used here was not be scaled to animal size and this might affect results (Lynn and Brown, 2010). However, some studies also show equally more anxiety in adolescent and adult rats (Macrì et al., 2002). In contrast, the idea that animals are most resilient against fearful events early in life as they have not learned to fear a specific situation because their amygdala is not yet involved in conditioned fear, therefore, they have no general aversion learned yet or again forgotten about it (for review see (Ganella and Kim, 2014)). So directly after weaning, rats may be least anxious as they tend to be explorative and fearless as soon as they can run by themselves.

Regarding USV, most studies focus on either pup vocalization in early postnatal days or adult vocalization with no studies investigating adolescent vocalization in anxious situations. More studies on adolescents are needed to fully understand the vocalization pattern in adolescence. Studies involving adolescents so far, focus on vocalization at 50 kHz frequencies in social interactions and play fighting (Kisko et al., 2015; Knutson et al., 2002). These studies find differences in vocalization between adolescent and adult rats. In one study, increased social interaction did not correlate with the number of USV emitted at 50 kHz frequencies in adolescent rats (Willey et al., 2009), underlining the different meaning of vocalization in adolescence. It seems that adolescent rats vocalize less than juvenile or adult rats in playful situations (Kisko et al., 2015; Schwarting, 2018). Adolescence is a time of immense neuronal change (Casey et al., 2008). Therefore, some researchers argue that rewarding situations like social interaction are differently rewarding for adolescent than for adult rats thus leading to different USV emissions (Willey and Spear, 2012). However, this study shows that adolescents also vocalize less during a test for anxiety. So, it might not only be rewarding situations that are being differently perceived but aversive situations as well. Interestingly, adolescence is a time of changes in affect regulation and the underlying neurobiology. More precisely, connections between the amygdala and prefrontal cortex form a circuitry that is involved in negative affect and which is important in mature affect regulation (Tottenham and Galván, 2016). However, these connections develop over a long period of time until adolescence or even adulthood with the amygdala circuitry being especially sensitive during adolescence (Tottenham and Galván,

2016). Even though the circuitry starts to form adult-like patterns regarding its connectivity in adolescence, the amygdala first shows hyper-reactivity to emotional stimuli indicating a time of change and instability in adolescence (Tottenham and Galván, 2016). Thus, it seems reasonable to assume that based on the changing neurobiology the reaction towards anxious situations is changing as well in adolescence reflected in a reduced USV-emission. More studies in adolescent vocalization patterns are needed to fully understand the emerging differences.

Conclusion

Adolescence is a time of neuronal change that might also affect USV in aversive situations. Even though no difference in anxiety behavior was observed in adolescents compared to adults, adolescents emitted significantly fewer calls compared to both, juvenile and adult rats. This finding underlines a different communication pattern in adolescence. USV investigation still holds potential for the study of affective disorders but deeper insights into adolescent communication in aversive situations is needed.

Acknowledgments

We thank Prof. Brudzynski for fruitful discussions, and Prof. Vos from the Ruhr-University Bochum and Ms Schmidt from WIGUM GmbH for enabling trouble-free use of Batloggers.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

A.M. and I.B. designed the study. A.M. and I.B. performed the experiments. The manuscript was written by A.M., I.B. and N.F. All authors approved the manuscript. This manuscript is our original work and it is submitted for first publication.

Abbreviations

EPM: Elevated plus maze

MANOVA: Multivariate analysis of variance

PND: Postnatal day

USV: Ultrasonic vocalization

References

Blakemore, S.-J., 2012. Development of the social brain in adolescence. J. R. Soc. Med. 105, 111–116. https://doi.org/10.1258/jrsm.2011.110221

Branchi, I., Santucci, D., Alleva, E., 2001. Ultrasonic vocalisation emitted by infant rodents: A tool for assessment of neurobehavioural development. Behav. Brain Res. 125, 49–56. https://doi.org/10.1016/S0166-4328(01)00277-7

Brudzynski, S.M., 2015. Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate. Curr. Neuropharmacol. 13, 180–192.

Burgdorf, J.S., Brudzynski, S.M., Moskal, J.R., 2020. Using rat ultrasonic vocalization to study the neurobiology of emotion: from basic science to the development of novel therapeutics for affective disorders. Curr. Opin. Neurobiol. 60, 192–200. https://doi.org/10.1016/j.conb.2019.12.008

Casey, B.J., Getz, S., Galvan, A., 2008. The adolescent brain. Dev. Rev. DR 28, 62–77. https://doi.org/10.1016/j.dr.2007.08.003

Demaestri, C., Brenhouse, H.C., Honeycutt, J.A., 2019. 22 kHz and 55 kHz ultrasonic vocalizations differentially influence neural and behavioral outcomes: Implications for modeling anxiety via auditory stimuli in the rat. Behav. Brain Res. 360, 134–145. https://doi.org/10.1016/j.bbr.2018.12.005

Drugan, R.C., Christianson, J.P., Stine, W.W., Soucy, D.P., 2009. Swim stress-induced ultrasonic vocalizations forecast resilience in rats. Behav. Brain Res. 202, 142–145. https://doi.org/10.1016/j.bbr.2009.03.021

Galván, A., 2010. Adolescent Development of the Reward System. Front. Hum. Neurosci. 4. https://doi.org/10.3389/neuro.09.006.2010

Ganella, D.E., Kim, J.H., 2014. Developmental rodent models of fear and anxiety: from neurobiology to pharmacology. Br. J. Pharmacol. 171, 4556–4574. https://doi.org/10.1111/bph.12643

Gee, D.G., Humphreys, K.L., Flannery, J., Goff, B., Telzer, E.H., Shapiro, M., Hare, T.A., Bookheimer, S.Y., Tottenham, N., 2013. A Developmental Shift from Positive to Negative Connectivity in Human Amygdala–Prefrontal Circuitry. J. Neurosci. 33, 4584–4593. https://doi.org/10.1523/JNEUROSCI.3446-12.2013

Jelen, P., Soltysik, S., Zagrodzka, J., 2003. 22-kHz Ultrasonic vocalization in rats as an index of anxiety but not fear: behavioral and pharmacological modulation of affective state. Behav. Brain Res. 141, 63–72. https://doi.org/10.1016/S0166-4328(02)00321-2

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Merikangas, K.R., Walters, E.E., 2005. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch. Gen. Psychiatry 62, 593–602. https://doi.org/10.1001/archpsyc.62.6.593

Kikusui, T., Nishizawa, D., Takeuchi, Y., Mori, Y., 2003. Conditioned fear-related ultrasonic vocalizations are emitted as an emotional response. J. Vet. Med. Sci. 65, 1299–1305. https://doi.org/10.1292/jvms.65.1299

Kisko, T.M., Euston, D.R., Pellis, S.M., 2015. Are 50-khz calls used as play signals in the playful interactions of rats? III. The effects of devocalization on play with unfamiliar partners as juveniles and as adults. Behav. Processes 113, 113–121. https://doi.org/10.1016/j.beproc.2015.01.016

Knutson, B., Burgdorf, J., Panksepp, J., 2002. Ultrasonic vocalizations as indices of affective states in rats. Psychol. Bull. 128, 961–977. https://doi.org/10.1037/0033-2909.128.6.961

Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10, 434–445. https://doi.org/10.1038/nrn2639

Lynn, D.A., Brown, G.R., 2010. The ontogeny of anxiety-like behavior in rats from adolescence to adulthood. Dev. Psychobiol. 52, 731–739. https://doi.org/10.1002/dev.20468

Macrì, S., Adriani, W., Chiarotti, F., Laviola, G., 2002. Risk taking during exploration of a plus-maze is greater in adolescent than in juvenile or adult mice. Anim. Behav. 64, 541–546. https://doi.org/10.1006/anbe.2002.4004

Oliveira, A.R., Barros, H.M.T., 2006. Ultrasonic Rat Vocalizations During the Formalin Test: A Measure of the Affective Dimension of Pain? Anesth. Analg. 102, 832–839. https://doi.org/10.1213/01.ane.0000196530.72813.d9

Sadananda, M., Wöhr, M., Schwarting, R.K.W., 2008. Playback of 22-kHz and 50-kHz ultrasonic vocalizations induces differential c-fos expression in rat brain. Neurosci. Lett. 435, 17–23. https://doi.org/10.1016/j.neulet.2008.02.002

Sánchez, C., 2003. Stress-induced vocalisation in adult animals. A valid model of anxiety? Eur. J. Pharmacol., Animal Models of Anxiety Disorders 463, 133–143. https://doi.org/10.1016/S0014-2999(03)01277-9

Schwarting, R.K.W., 2018. Ultrasonic vocalization in juvenile and adult male rats: A comparison among stocks. Physiol. Behav. 191, 1–11. https://doi.org/10.1016/j.physbeh.2018.03.023

Stansfield, K.H., Kirstein, C.L., 2006. Effects of novelty on behavior in the adolescent and adult rat. Dev. Psychobiol. 48, 10–15. https://doi.org/10.1002/dev.20127

Tottenham, N., Galván, A., 2016. Stress and the Adolescent Brain: Amygdala-Prefrontal Cortex Circuitry and Ventral Striatum as Developmental Targets. Neurosci. Biobehav. Rev. 70, 217–227. https://doi.org/10.1016/j.neubiorev.2016.07.030

Walf, A.A., Frye, C.A., 2007. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat. Protoc. 2, 322–328. https://doi.org/10.1038/nprot.2007.44

Willey, A.R., Spear, L.P., 2012. Development of anticipatory 50kHz USV production to a social stimuli in adolescent and adult male Sprague-Dawley rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2011.10.001

Willey, A.R., Varlinskaya, E.I., Spear, L.P., 2009. Social interactions and 50 kHz ultrasonic vocalizations in adolescent and adult rats. Behav. Brain Res. 202, 122–129. https://doi.org/10.1016/j.bbr.2009.03.025

Asymmetry of turning behavior in rats is modulated by early life stress

Annakarina Mundorf *[a], Hiroshi Matsui [b], Sebastian Ocklenburg [b] and Nadja Freund [a]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany

[b] Institute of Cognitive Neuroscience, Department Biopsychology, Ruhr University Bochum, Germany

*Corresponding author

Research aritcle / Minor Revision (2020) Behav. Brain Res.

Abstract

Atypical leftward behavioral asymmetries have been associated with early life stress (ELS) and psychopathologies in humans and animals. Maternal separation (MS) is a frequently used model to investigate ELS and psychopathologies but has not yet been studied in terms of asymmetries. This study aims to investigate whether prolonged MS induces atypical leftward asymmetries in the turning behavior of rats. MS was performed from postnatal days 2-20 followed by a second stressor from postnatal days 21-40. Asymmetry of turning behavior was then examined in the elevated plus-maze test upon weaning (juveniles and dams) or adolescence. The number of left and right turns was calculated per animal using the deep learning software package DeepLabCut enabling markerless pose estimation. Then, a lateralization quotient (LQ) was determined for each animal allowing to investigate the strength as well as the preferred side of asymmetry. LQ analysis revealed a significant leftward asymmetry in the prolonged stress group. Moreover, analyzing the number of turns revealed significantly more left than right turns in total in this group. Control animals showed no asymmetries in turning behavior. These results indicate that prolonged stress during the early postnatal days led to atypical leftward turning behavior. The stress-induced atypical asymmetry might be a mediator of ELS and the development of psychiatric disorders.

Keywords: maternal separation, lateralization, heritability, leftward, Sprague-Dawley, DeepLabcut

1. Introduction

Hemispheric asymmetries are a general principle of functional organization in the vertebrate brain and have been reported for many behaviors and cognitive systems [1–7]. In both humans and animals, it has been shown that genetic and non-genetic factors are involved in the ontogenesis of hemispheric asymmetries [8–14]. A recent study utilizing the UK Biobank dataset found that in humans, several early life factors significantly contributed to left-handedness [15]. These factors included the year and location of birth, birthweight, being part of a multiple birth, season of birth, breastfeeding, and sex.

As both low birthweight and being part of a multiple birth are potentially linked to birth stress, it is conceivable, that early life stress (ELS), e.g. via hormonal effects, could affect the ontogenesis of hemispheric asymmetries. Indeed, it has been shown that in humans and other species, there are relations between different forms of stress (ELS, chronic stress and acute stress), but the direction of these relations do not follow a consistent pattern [16]. One problem here might be that in research in human participants, experimental variation of ELS conditions is impossible due to obvious ethical reasons. Therefore, all analyses are usually post-hoc, e.g. by comparing data on early life events from medical records between different individuals and linking them to hemispheric asymmetries as adults. While there are studies on the effects of ELS and maternal separation (MS) in specific human populations, e.g. in the English and Romanian Adoptees (ERA) study [17], these works, unfortunately, do not include data on hemispheric asymmetries.

Therefore, research in animal models that induce ELS in a controlled manner is necessary to investigate the consequences of ELS on brain asymmetry. One well-established and widely used animal model to investigate the consequences of ELS is MS [18]. However, as the MS procedure varies across studies, e.g. in time being separated daily and number of days MS is conducted, the results can be inconsistent [19, 20]. Therefore, an already established MS protocol was used that consists of daily separation for 4 hours over the first postnatal weeks

(postnatal day 2-20) which leads to increased depression-like behavior in adolescent female and male Sprague-Dawley rats [21]. To investigate the consequences of post-weaning chronic stress exposure, social isolation is a frequently used paradigm that is also known to induce anxiety- and depression-like phenotypes [22, 23]. Using both stress paradigm allows investigating different stress-sensitive windows (early and late childhood) separately as well as an effect of both stressors experienced consecutively.

Neuronal asymmetries are also well-studied in the rat. Researchers have found hemispheric dopaminergic asymmetries in the medial prefrontal cortex matching the rats turning asymmetry after ethanol injection (right turning rats had activation in the right hemisphere) [24] as well as dominant right prefrontal cortex activation in stress regulation [25]. It is known that rats, as well as other rodents, have an intrinsic side preference and that this preference is modulated by dopamine release [26]. More precisely, rodents turn in the direction contralateral to the striatum side containing more dopamine. This behavior can be modulated by e.g. amphetamine and potentiated into circling behavior [26]. In a genetically modified circling rat, researchers found lower striatal dopamine ipsilateral to the preferred rotation side [27]. Whereas stress exposure led to an increase of dopamine release in the contralateral striatum in modified rats but not in wildtype rats [27]. Neonatal novelty exposure using the novel cage test during the first 3 weeks of life led to a developmental stable turning bias [28]. Turning behavior was defined as 90° rotation and was analyzed during a 5 minutes novel cage test. Then, the lateralization score was calculated showing a right-shift in turning bias in the novel cage in males but not females [28]. However, most studies investigating asymmetry behavior in rats find a favor of the right side e.g. when testing turning behavior in mazes [29]. Moreover, they show strong lateralization of the individual but no side bias at population level in head turning [30] and paw preference (54% right sided) [31]. Interestingly, when investigating head turning asymmetry, left turning rats showed increased behavioral despair in the forced swim test than right turning rats [30].

As mentioned, asymmetries can also be assessed in behavior. As in humans, there are different behaviors to analyze laterality in rats. Atypical lateralization in rodents can be investigated by analyzing e.g. paw preference [31], head-turning [30] or general body turning behavior [24, 27–29]. The benefit in analyzing general turning behavior in a setup where the animal can move freely is that turning behavior occurs more naturally. Therefore, when analyzing turning behavior in the rat, several behavioral tests such as the open field, the T-maze and the elevated plus maze (EPM) have proven to be very useful as animals have to turn frequently to navigate through the maze [29].

A recently published review investigated the role of stress in psychiatric disorders and atypical lateralization [32]. It was concluded, that ELS as well as chronic hypothalamic-pituitary-adrenal (HPA)-axis elevation change structural and functional asymmetries [32]. More precisely, an acute or chronic increase in glucocorticoids impacts the corpus callosum (which is important for interhemispheric communication) leading to altered hemispheric asymmetries [32]. Thus, an increased stress exposure would lead to increased glucocorticoid levels, and consequently to more atypical asymmetries. These atypical asymmetries were also observed in psychiatric patients [32].

As mentioned above, hemispheric asymmetries are especially vulnerable to ELS. Exposing rats to chronic stress early in life should shift the hemispheric asymmetry compared to controls. This shift in hemispheric asymmetry can then be observed in altered turning behavior. To investigate whether different times of ELS exposure have a different impact on asymmetric turning behavior, animals were either exposed to MS in the early postnatal days or to isolation in late childhood. For the consequences of a prolonged chronic stress exposure, one group of animals was subjected to MS followed by isolation. Consequences should be most obvious in this group.

Besides modulation via stress exposure, some behavioral asymmetries have also been found to be hereditary in humans [33, 34]. Whereas studies in inbred mice did not show any

heritability of paw preference [35, 36]. However, as not all behavioral asymmetries are inherited, another explanation for this transgenerational modulation of asymmetry might be via epigenetic mechanisms [14]. This is particularly interesting regarding effects of maternal (or intrauterine) stress exposure on offspring asymmetries [14]. It is also possible, that some asymmetries are controlled by genetic and others by environmental factors [33, 34].

In this study, rats were exposed to MS, isolation or both and compared to controls. Turning behavior was analyzed during a 5 minutes EPM test to investigate turning asymmetries after stress exposure. Moreover, dams were analyzed as well, allowing for a mother-offspring regression analysis to investigate whether maternal turning behavior could predict offspring turning behavior. To our knowledge, this is the first study to investigate asymmetries in turning behavior in an animal model for MS and isolation.

2. Methods

2.1 Animals

Animals were housed under standard conditions (22± 2°C room temperature, 55 ± 25 % humidity) and standard lighting (12h/12h) with free access to water and food. Experiments were conducted under the principles of Germany's Animal Welfare Act after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Northrhine-Westfalia). 16 timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were single housed upon arrival and were divided into the MS (N = 8) or control group (CG, N = 8). Postnatal day (PND) 0 was equal to the day of birth of the pups. At PND2 pups were sexed and culled to 10, if possible five male and five female pups leading to a total of 79 female and 78 male pups.

2.2 Stress induction

In total, animals were assigned to 4 different groups. The MS group, the CG, the isolation stress (IS) group and both MS and IS (MSIS) group. Besides stress exposure, 3 different age groups tested emerge as follows: dams, juvenile rats (PND21) and adolescent rats (PND41).

MS was carried out as previously described [21]. In brief, pups were separated from their mother and siblings from PND2 – PND 20 for 4 hours every day during the dark (= active) phase. Pups were placed in separate cages (for the early days of separation, pups were placed on a heating mat adjusted to 37°C, pups were divided by a self-made plastic grid which allowed placing home cage bedding on top of the heating mat). At PND 21 all pups were weaned. One animal per litter and sex was tested in the EPM on the day of weaning as well as the dams. The littermates were either group-housed (GC) or single-housed (IS) until PND42. All animals were then tested in the EPM. Single housing is considered a stressor. Therewith, animals in the IS group, that were not subjected to MS before, received a late stressor instead. Animals in the IS group, that were already exposed to MS, were exposed to a second stressor (MSIS). Animals placed in group-housing were either only subjected to MS or received no stress at all (GC). Therewith, the different groups are composed as follows. Thus overall, the following groups were analyzed:

Mothers

- CG: $N = 8$
- MS: $N = 8$

Offspring PND21:

- CG: $N = 8$ females and 8 males
- MS: $N = 8$ females and 8 males

Offspring PND41:

- CG: $N = 24$ females and 24 males
- MS: $N = 23$ females and 22 males

- IS: *N* = 8 females and 8 males
- MSIS: *N* = 8 females and 8 males

2.3 Analysis of turning behavior asymmetries

Behavioral testing was carried out in the dark phase under red light. The EPM consists of two open arms (50 cm x 10 cm) and two closed arms (50 cm x 10 cm) and a center connecting the arms. The maze is adjusted at 50 cm in height. Animals were placed on the EPM facing a closed arm and could discover the maze freely for 5 minutes while being filmed using an HD Webcam (C920 Pro, Logitech) connected to a laptop. A video-based offline tracking was performed via python software 'DeepLabCut', [37]. Horizontal x-y coordinates of the head, the body center, and the tail base were extracted, and smoothed with a median filter to remove noise. Body and head orientation were determined as vectors from tail to body, and from body to head, respectively. Turning behavior was defined as turning the head more than 45 degrees from the body center which was then counted across a session.

2.4 Statistical analysis

Dams and offspring were analyzed separately, as stress exposure was either early in life (offspring) or in the sensitive postpartum time (dams). Data in dams was analyzed using 2 × 2 repeated-measures ANOVA with the within-subjects' factors side (left turning, right turning) and the between-subjects' factors group (CG, MS). Data in offspring was analyzed using 2 × 4 repeated-measures ANOVA with the within-subjects' factors side (left turning, right turning) and the between-subjects' factors group (CG, MS, IS, MSIS). Post-hoc test was corrected for multiple comparisons by using Bonferroni correction. Additionally, a lateralization quotient (LQ) was determined for each animal following the formula LQ= ((R– L) / (R + L)) ×100, with R indicating the number of right turns and L indicating the number of left turns. The LQ ranges between -100 and 100, with positive values representing a right-sided turning bias and negative values indicating a left-sided turning bias. Moreover, we also assessed individual side

preferences independent of the strength of lateralization by classifying animals as being right-preferent (positive LQ) or left-preferent (negative LQ).

In order to be able to estimate heritability, LQ data from mothers were used to predict LQ data from offspring using linear regressions. Heritability (h^2) in this model is equivalent to the regression coefficient b of the parent-offspring regression [38].

3. Results

3.1 Asymmetries in dams

Since the overall *N* was low in dams (*N* = 16) we tested the data for normal distribution using KS tests in order to decide whether parametric testing would be possible. Both, the amount of left-sided (p = .98) and right-sided turning behavior (p = 1.00) did not significantly deviate from normal distribution. Therefore, we used a 2 × 2 repeated-measures ANOVA with the within-subjects' factors side (left turning, right turning) and the between-subjects' factors group (CG, MS) to investigate the data in dams. Both, the main effect of side ($F_{(1,14)} = .42$; p = .53) and the main effect of group ($F_{(1,14)} = .01$; p = .95) failed to reach significance. The interaction side × group approached significance ($F_{(1,14)} = 3.56$; p = .08) and tentatively suggested that animals in the MS condition showed a more rightward asymmetry (number of left turns: 23.88 +/- 7.77 SD; number of right turns: 30.50 +/- 7.29) than animals in the CG (number of left turns: 28.62 +/- 8.86; number of right turns: 25.38 +/- 5.93). However, since the effect failed to reach significance, this relation is at best weak.

3.2 Asymmetries in offspring

Figure 1 shows the distribution of left turns and right turns in the four offspring groups. We conducted a 2 × 4 repeated-measures ANOVA with the within-subjects' factors side (left turning, right turning) and the between-subjects' factors group (CG, MS, IS, MSIS) to investigate the data in offspring. Both, the main effect of side ($F_{(1,152)} = 2.46$; p = .11) and the main effect of group ($F_{(1,153)} = 2.30$; p = .08) failed to reach significance. However, the

interaction side × group reached significance ($F_{(3,153)} = 2.93$; $p = .036$; partial $\eta^2 = .05$). To further investigate this effect, we conducted Bonferroni-corrected post-hoc tests.

Post-hoc tests failed to reach significance for the CG (number of left turns: 28.97 +/- 12.47; number of right turns: 27.00 +/- 11.94; p = .20), MS group (number of left turns: 28.28 +/- 12.10; number of right turns: 29.79 +/- 9.69; p = .33), and IS group (number of left turns: 32.31 +/- 11.04; number of right turns: 33.25 +/- 11.50; p = .76). In contrast, the post-hoc test reached significance for the MSIS group (p = .009), indicating significantly more left turns (37.88 +/- 10.73) than right turns (29.88 +/- 7.21) in this group.

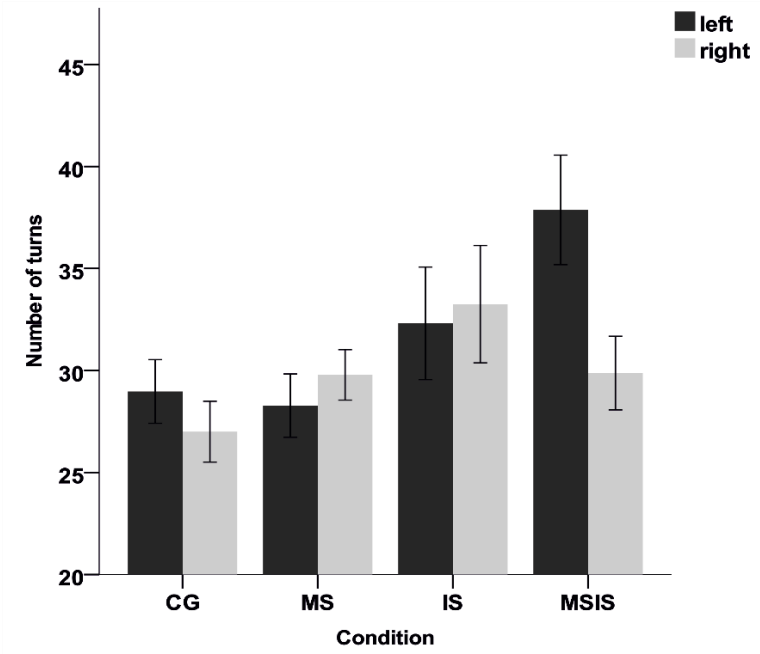


Figure 1: Distribution of left turns and right turns in the four offspring groups. Mean number of left (black) and right (grey) turns per group ± SD is shown. MSIS animals show significantly more left turns (37.88 +/- 10.73) than right turns (29.88 +/- 7.21).

To further analyze this effect independently of individual reaction rates, we calculated one-sample t-tests against zero for the LQ in all four conditions (see Figure 2). This effect failed to reach significance for the CG ($t_{(63)} = -1.24$; $p = .22$), the MS group ($t_{(60)} = 1.51$; $p = .14$), and the IS group ($t_{(15)} = .17$; $p = .87$). However, in the MSIS condition, there was a significant leftward asymmetry (LQ = -11.28 +/- 15.89; $t_{(15)} = -2.84$; $p = .012$).

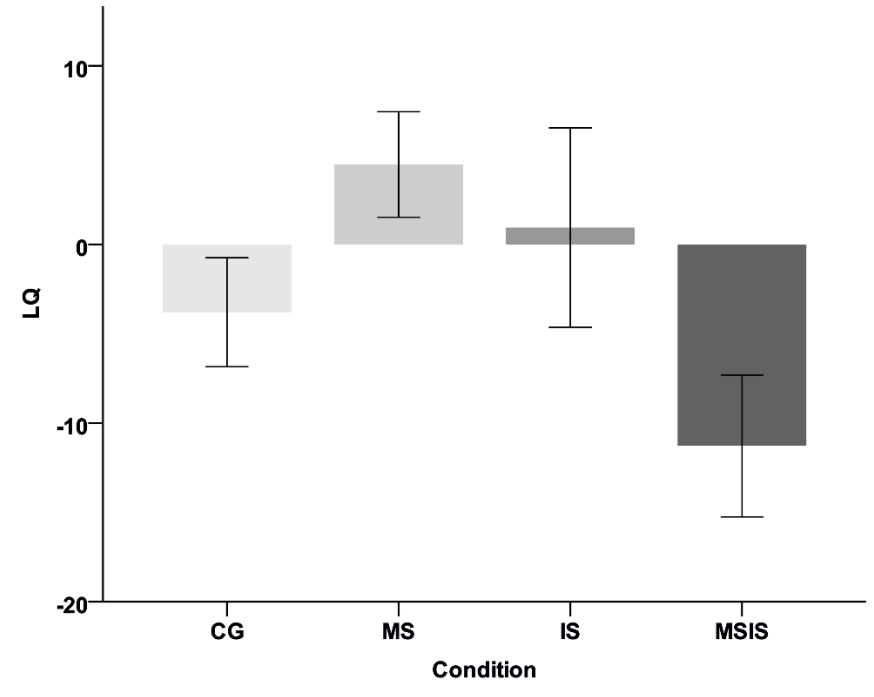


Figure 2: Lateralization quotient (LQ) in the four offspring groups. Positive values represent a right-sided turning bias and negative values indicate a left-sided turning bias. MSIS animals show a significant leftward asymmetry (LQ = -11.28 +/- 15.89; $t_{(15)} = -2.84$; p = .012).

In order to test whether there were any differences in the side of preferences independent of the strength of lateralization, we classified animals as being right-preferent (positive LQ) or left-preferent (negative LQ) and used a non-parametric Kruskal-Wallis test to determine differences in the distribution of these categories between the four groups. The effect reached significance ($\chi^2_{(3)} = 12.23$, p = .007), indicating that the four groups showed different distributions of left- and right-preferent animals (see table 1)

Table 1: Number of left-preferent and right-preferent animals in the four offspring groups.

		Left side	**Right side**	
Group	**CG**	38	26	64
	MS	23	38	61
	IS	7	9	16
	MSIS	13	3	16
Overall		81	76	157

We then compared the groups directly with each other using Mann-Whitney U-tests. Here it was shown that the MS group had a significantly more rightward distribution than the CG (U = 1529, p = .016). The other two stress groups did not differ from the CG (all p's > .10). Moreover, the MSIS group had a significantly more leftward distribution than the MS group (U = 275, p = .002).

In the last step, we used an univariate ANOVA with absolute LQ's in order to test whether there were any differences in the strength of preferences independent of the side of lateralization. However, the effect failed to reach significance ($F_{(3,153)} = .61$; p = .61).

3.3 Heritability

In order to investigate, whether hemispheric asymmetries in parents predicted hemispheric asymmetries in offspring, we used LQ parent-offspring regressions (see table 2). Parent-offspring regressions failed to reach significance in all groups (all p's > .24) and heritability quotients were generally low (all $h^2 < .16$).

Table 2: LQ parent-offspring regressions for the different groups.

Group	**R^2**	**F**	**h^2/b**	**T**
All offspring	0.008	$F_{(1,155)} = 1.27$; p = 0.26	0.90	T = 1.12; p = 0.26
CG	0.003	$F_{(1,62)} = 1.65$; p = 0.69	0.05	T = 0.41; p = 0.69
All EG	0.004	$F_{(1,91)} = 0.38$; p = 0.54	0.07	T = 0.62; p = 0.52
MS	0.02	$F_{(1,59)} = 1.33$; p = 0.25	0.15	T = 1.15; p = 0.25
IS	0.0003	$F_{(1,14)} = 0.004$; p = 0.95	0.02	T = 0.06; p = 0.95
MSIS	0.04	$F_{(1,14)} = 0.52$; p = 0.48	-0.19	T = -0.72; p = 0.48

4. Discussion

The aim of the present study was to investigate the effect of ELS on behavioral asymmetries in a rodent model. Turning asymmetry was investigated after MS, IS, MSIS or no stress (CG). Only MSIS led to a significant leftward shift in turning asymmetry compared to no turning asymmetry in CG. MS exposure of dams during the post-partum time did not affect turning behavior. However, a rightward trend was observed in MS dams similar to MS offspring. The turning behavior of dams was not predictive of offspring turning behavior.

Most studies in rats found no turning bias on a population level in the control groups even though the individuals are strong and stable lateralized [29–31]. The finding of chronic stress-induced atypical asymmetry is in line with previous findings indicating a shift of behavioral asymmetry to the left side. As shown in human and animal studies, stress exposure often leads to greater activation of the right hemisphere [16] resulting in more leftward behavioral asymmetry. These altered hemispheric asymmetries have repeatedly been associated with psychiatric disorders [39–44]. For example, studies on frontal electroencephalograph (EEG) alpha oscillation asymmetries in schizophrenia patients found consistent relative left-sided resting frontal alpha power in patients instead of equally distributed in healthy humans [44, 45]. Greater left-sided resting EEG alpha indicates reduced activity in the left hemisphere [44]. In healthy humans, a greater left frontal activity is associated with positive emotion and approach behavior whereas a greater right frontal activity to negative emotion or avoidance behavior such as withdrawal [46]. Handedness, as a behavioral measurement of asymmetries, has been most extensively studied in schizophrenia. Here, several meta-analyses confirmed a more than 1.5 fold increased odds ratio for non-right-handedness (left-handedness and mixed-handedness) in people diagnosed with schizophrenia compared to healthy controls [43, 47]. These studies underline the association of atypical functional and behavioral lateralization and psychiatric disorders. Of note, a study investigating left-handedness and depression in children found a significantly higher prevalence of depression in left-handed children [48]. As in this

study, juvenile and adolescent offspring were analyzed, the results in atypical turning behavior are in line with higher prevalence of depression in left-handed children [48]. The atypical leftward asymmetry found especially in schizophrenia and depression as well as after ELS might be explained by the valence hypothesis. Regarding that hypothesis, the right-hemisphere is associated with negative emotion whereas the left hemisphere with positive emotion [49].

Lesion studies in humans revealed different hemispheric functions of emotion processing with the left frontal hemisphere controlling positive emotions triggering approach behavior whereas the right frontal hemisphere is responsible for negative emotions and avoidance behavior [50]. Damage to one hemisphere reduced the emotionality respectively and thus proving hemispheric control of emotion [50]. This emotional lateralization is found across all vertebrates, including domestic and non-domestic animals as well as primates and humans. Emotions as fear/anxiety and aggression always activate the right hemisphere except in fish, probably as fish are not responding emotionally to presented stimuli. The reaction to food reward, on the other hand, is dominated by the left hemisphere across species [51]. For example, chicks and adult hens both show a left eye preference when observing aerial predators [52, 53]. The same left-side preference was found in common wall lizards [54, 55] and in domestic cattle herds (when observing novel stimuli) [56]. Dogs confronted with a dominant dog (fear-inducing stimuli) show more leftward tail wagging [57]. Lesion studies in mice and rats underline this asymmetric side preference in emotional situations. In mice, a right-hemispherectomy led to an increase in immobility time in the forced swim test [58]. In a study inducing side specific lesions of the medial prefrontal cortex in rats, only right or bilateral lesion reduced stress-induced cortisol levels [59]. To sum up, asymmetric emotion processing is consistent and stable across animals all showing right-hemispheric control of negative emotions expressed in leftward behavioral asymmetries.

When investigating the human stress response and asymmetric cerebral hemispheres, some researchers suggest that the cerebral stress response, including the HPA axis, is controlled

mainly by the right hemisphere [60]. Aversive situations evoke a stress response and consequently negative emotions [61]. Prolonged and unavoidable exposure to aversive situations leads to an adaptation of the organism potentially resulting in passive behavior or learned helplessness. Administering antidepressants reverse this behavioral effect in rats [61]. Thus, experiencing stress leads to negative emotions that trigger avoidance behavior in rats. As emotional processing is asymmetric, a chronic increased right hemisphere activation results in more left-sided behavior [16].

Interestingly, MS or IS alone was not enough to induce significant altered behavioral asymmetries. It might be possible that a high level of chronic stress is needed before an altered asymmetry manifests in behavior. Children with lower birth weight show more lateralized hemispheric blood flow when exposed to acute stress than children with a normal birth weight exposed to acute stress [62]. Acute stress exposure in healthy humans leads to more negative effects when presented with emotional faces [63]. Stressed participants showed a faster response for angry faces when occurring in the left visual hemisphere than non-stressed participants [63]. Both studies indicate that overall higher stress exposure might lead to more lateralized hemispheric functioning. Similar results have been found in patients suffering from posttraumatic stress disorder (PTSD). In a study investigating children suffering from PTSD and handedness compared to healthy controls, PTSD symptom severity significantly correlated positively with mixed laterality [40]. In an EEG study with adult PSTD patients and controls, left-sided frontal activation was associated with less emotionally intense reactions to negative stimuli in PTSD patients [39]. Another study in solders revealed that high levels of childhood trauma together with might levels of PTSD symptoms were associated with greater frontal activation asymmetries, depending on symptoms respectively [64]. Recently it has been shown that mice have facial expressions of their emotional state that are associated with neuronal correlates of emotion [65]. More interestingly, the study revealed that emotions depend on an innate or learned value in mice as well and thus are altered by experience or circumstances (e.g.

a thirsty mouse has more pleasure when drinking) [65]. Therefore, a situation that is experienced to elicit negative emotions might trigger a stronger negative reaction when reexperienced (second stressor). As rats exposed to MSIS experience two consecutive stressors, their emotional state might be more aversive compared to only MS or IS rats, leading to stronger activation of the right hemisphere.

Elevated levels of steroids, such as glucocorticoids, might influence cerebral asymmetries as well by altering callosal communication [16, 66]. Glucocorticoids are important for normal brain maturation [67]. But exposure to elevated glucocorticoid levels of the fetal brain modifies the HPA axis function and stress reaction for a lifetime [68]. Of note, elevated glucocorticoid levels and HPA activity are also associated with depression [69]. Prenatal stress exposure in humans by e.g. maternal factors or birth complications are already known to influence the ontogenesis of behavioral asymmetries [70]. As rats are born prematurely [68] the maturation of the brain in the early postnatal days of rats is comparable to prenatal human brain maturation. ELS is also associated with a greater risk to develop psychiatric disorders like depression [71].

Regarding mother-offspring regression, the heritability of LQ's generally was low and parent-offspring regressions for LQ's failed to reach significance in all groups. This is consistent with the existing literature on the heritability of hemispheric asymmetries on the behavioral level in rodents. In studies investigating handedness (paw preference) in mice, no genetic influence could be established [35, 36]. When analyzing 3 generations of selected inbred mice for paw preference revealed stable preferences for one paw in different behavioral tasks [35]. Moreover, both left and right paw preferences were observed in a genetically uniform inbred population [35]. By then only mating left-handed female inbred mice with left-handed male mice and right-handed mice respectively, as well as mixed handed mating pairs, researchers were able to investigate the heritability of paw preference in mice [36]. The results show a clear preference for one paw in most mice but no influence of genetic predisposition

[36]. Therefore, environmental influence on behavioral lateralization is expected [35, 36, 70]. So far, epigenetic mechanisms are thought to be a modulation factor in parental influenced lateralization patterns [70]. In humans, however, some behavioral asymmetries such as handedness [34] and cognitive factors [33] show high heritability. Therefore, it seems possible, that some lateralized functions are controlled by genetic factors whereas others might be regulated by environmental influences [33, 70].

Generally, there are two different genetic pathways discussed to determine the direction and strength of brain asymmetry [1]. The FGF signaling pathway is responsible for the general development of brain asymmetries [72]. Whereas the Nodal signaling pathway determines the direction of brain asymmetries [73]. In this study, the effect of laterality was driven by the direction of asymmetry (preferred side) but not by the strength of asymmetry, namely more left-sided rats after stress exposure. Therefore, one might speculate that ELS might disrupt the Nodal signaling pathway and thus potentially affects the direction of laterality rather than the already established strength of brain asymmetry.

Our results underline the findings of atypical leftward behavioral asymmetry after stress exposure. In line with previous studies, we found atypical leftward behavior after prolonged chronic stress. Given that ELS is a risk factor to develop psychiatric disorders, hemispheric asymmetries altered by ELS might be a mediator for the development of psychiatric disorders as well. More interestingly, hemispheric and behavioral asymmetries might serve as applicable biomarkers for high-risk individuals before clinical symptoms manifest. Of course, more studies are needed to prove its validity for diagnostic use.

In this study, no corticoid levels were measured. Therefore, the analysis of the individual actual stress level was not possible and thus no correlation of turning asymmetry and corticoid level could be made. Moreover, only turning behavior was analyzed. Other lateralized behaviors might be more susceptible to MS or IS. Futures studies should, therefore, investigate the consequences of early stress exposure at several developmental stages until adulthood.

Including multiple behavioral tests like paw preferences, turning behavior, and head-turning behavior will allow assessing more behavioral asymmetries. Furthermore, corticoid levels should be assessed. As blood sampling to e.g. measure corticoid levels in animals induce stress as well sampling should occur before testing. Investigating hemispheric asymmetries as well will also allow a more precise insight into neuronal correlates of altered behavioral asymmetries.

Conclusion

Chronic stress exposure during early life leads to atypical leftward asymmetry in turning behavior in an animal model of ELS. Moreover, as the turning behavior of mothers was not predictive for offspring it might be controlled by environmental rather than genetic factors. Analyzing turning behavior after stress proves to be a good model to investigate atypical leftward asymmetries observed in psychiatric patients. Analyzing hemispheric and behavioral asymmetries could serve as applicable biomarkers for high-risk individuals before clinical symptoms manifest. More studies are needed to prove its validity first.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

A.M. designed the study. A.M. performed the animal experiments. H.M. performed the data analysis and was supported by O.S. The manuscript was written by A.M., H.M., S.O. and N.F. All authors approved the manuscript. This manuscript is our original work and it is submitted for first publication.

Abbreviations

CG: Control group
EEG: Electroencephalograph
ELS: Early life stress
ERA: English and Romanian Adoptees

HPA: Hypothalamic-pituitary-adrenal
IS: Isolation stress
LQ: Lateralization quotient
MS: Maternal separation
MSIS: Maternal separation + isolation stress
PTSD: Posttraumatic stress disorder

References

[1] Güntürkün O, Ocklenburg S. Ontogenesis of Lateralization. Neuron 2017;94:249–63.
[2] Ocklenburg S, Güntürkün O. The lateralized brain: The neuroscience and evolution of hemispheric asymmetries. London: Academic Press; 2018.
[3] Rogers LJ. Asymmetry of brain and behavior in animals: Its development, function, and human relevance. Genesis (New York, N.Y. 2000) 2014;52:555–71.
[4] Rogers LJ. Asymmetry of brain and behavior in animals: Its development, function, and human relevance. Genesis (New York, N.Y. 2000) 2014;52:555–71.
[5] Rogers LJ. Manual bias, behavior, and cognition in common marmosets and other primates. Progress in brain research 2018;238:91–113.
[6] Stancher G, Sovrano VA, Vallortigara G. Motor asymmetries in fishes, amphibians, and reptiles. Progress in brain research 2018;238:33–56.
[7] Vallortigara G, Rogers LJ. A function for the bicameral mind. Cortex; a journal devoted to the study of the nervous system and behavior 2020;124:274–85.
[8] Duboc V, Dufourcq P, Blader P, Roussigné M. Asymmetry of the Brain: Development and Implications. Annual review of genetics 2015;49:647–72.
[9] Horstick EJ, Bayleyen Y, Burgess HA. Molecular and cellular determinants of motor asymmetry in zebrafish. Nature communications 2020;11:1170.
[10] Manns M, Ströckens F. Functional and structural comparison of visual lateralization in birds - similar but still different. Frontiers in psychology 2014;5:206.
[11] Medland SE, Duffy DL, Wright MJ, Geffen GM, Hay DA, Levy F et al. Genetic influences on handedness: data from 25,732 Australian and Dutch twin families. Neuropsychologia 2009;47:330–7.
[12] Medland SE, Duffy DL, Wright MJ, Geffen GM, Martin NG. Handedness in twins: joint analysis of data from 35 samples. Twin research and human genetics the official journal of the International Society for Twin Studies 2006;9:46–53.
[13] Ocklenburg S, Beste C, Arning L, Peterburs J, Güntürkün O. The ontogenesis of language lateralization and its relation to handedness. Neuroscience and biobehavioral reviews 2014;43:191–8.
[14] Schmitz J, Güntürkün O, Ocklenburg S. Building an Asymmetrical Brain: The Molecular Perspective. Frontiers in psychology 2019;10:982.
[15] Kovel CGF de, Carrión-Castillo A, Francks C. A large-scale population study of early life factors influencing left-handedness. Scientific reports 2019;9:584.
[16] Ocklenburg S, Korte SM, Peterburs J, Wolf OT, Güntürkün O. Stress and laterality - The comparative perspective. Physiology & behavior 2016;164:321–9.
[17] Rutter M, Sonuga-Barke EJ, Castle J. I. Investigating the impact of early institutional deprivation on development: background and research strategy of the English and Romanian Adoptees (ERA) study. Monographs of the Society for Research in Child Development 2010;75:1–20.

[18] Molet J, Maras PM, Avishai-Eliner S, Baram TZ. Naturalistic rodent models of chronic early-life stress. Dev Psychobiol 2014;56:1675–88.
[19] Lehmann J, Feldon J. Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? Rev Neurosci 2000;11:383–408.
[20] Freund N, Thompson BS, Denormandie J, Vaccarro K, Andersen SL. Windows of vulnerability: maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. Neuroscience 2013;249:88–97.
[21] Leussis MP, Freund N, Brenhouse HC, Thompson BS, Andersen SL. Depressive-Like Behavior in Adolescents after Maternal Separation: Sex Differences, Controllability, and GABA. Dev Neurosci 2012;34:210–7.
[22] Mumtaz F, Khan MI, Zubair M, Dehpour AR. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomedicine & Pharmacotherapy 2018;105:1205–22.
[23] Weiss IC, Pryce CR, Jongen-Rêlo AL, Nanz-Bahr NI, Feldon J. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behavioural brain research 2004;152:279–95.
[24] Nielsen DM, Crosley KJ, Keller RW, Glick SD, Carlson JN. Ethanol induced differences in medial prefrontal cortex dopamine asymmetry and in nucleus accumbens dopamine metabolism in left- and right-turning rats. Brain research 1999;823:207–12.
[25] Sullivan RM. Hemispheric Asymmetry in Stress Processing in Rat Prefrontal Cortex and the Role of Mesocortical Dopamine. Stress 2004;7:131–43.
[26] Glick SD, Zimmerberg B, Jerussi TP. Adaptive significance of laterality in the rodent. Annals of the New York Academy of Sciences 1977;299:180–5.
[27] Fedrowitz M, Potschka H, Richter A, Löscher W. A microdialysis study of striatal dopamine release in the circling rat, a genetic animal model with spontaneous lateralized rotational behavior. Neuroscience 2000;97:69–77.
[28] Akers KG, Reeb BC, Tang AC. Developmentally stable sex-dependent modulation of turning asymmetry by neonatal novelty exposure. Behavioural brain research 2004;155:257–63.
[29] Schwarting RKW, Borta A. Analysis of behavioral asymmetries in the elevated plus-maze and in the T-maze. Journal of neuroscience methods 2005;141:251–60.
[30] Soyman E, Yilmaz GD, Canbeyli R. Head-turning asymmetry: A novel lateralization in rats predicts susceptibility to behavioral despair. Behavioural brain research 2018;338:47–50.
[31] Cunha AM, Esteves M, das Neves SP, Borges S, Guimarães MR, Sousa N et al. Pawedness Trait Test (PaTRaT)-A New Paradigm to Evaluate Paw Preference and Dexterity in Rats. Front Behav Neurosci 2017;11:192.
[32] Berretz G, Wolf OT, Güntürkün O, Ocklenburg S. Atypical lateralization in neurodevelopmental and psychiatric disorders: What is the role of stress? Cortex; a journal devoted to the study of the nervous system and behavior 2020;125:215–32.
[33] Ocklenburg S, Ströckens F, Bless JJ, Hugdahl K, Westerhausen R, Manns M. Investigating heritability of laterality and cognitive control in speech perception. Brain and Cognition 2016;109:34–9.
[34] Lien Y-J, Chen WJ, Hsiao P-C, Tsuang H-C. Estimation of heritability for varied indexes of handedness. Laterality 2015;20:469–82.
[35] Collins RL. On the inheritance of handedness. I. Laterality in inbred mice. J Hered 1968;59:9–12.

[36] Collins RL. On the inheritance of handedness. II. Selection for sinistrality in mice. The Journal of heredity 1969;60:117–9.
[37] Nath T, Mathis A, Chen AC, Patel A, Bethge M, Mathis MW. Using DeepLabCut for 3D markerless pose estimation across species and behaviors. Nature Protocols 2019;14:2152–76.
[38] Visscher PM, Hill WG, Wray NR. Heritability in the genomics era--concepts and misconceptions. Nature reviews. Genetics 2008;9:255–66.
[39] Meyer T, Quaedflieg CWEM, Weijland K, Schruers K, Merckelbach H, Smeets T. Frontal EEG asymmetry during symptom provocation predicts subjective responses to intrusions in survivors with and without PTSD. Psychophysiology 2018;55.
[40] Saltzman KM, Weems CF, Reiss AL, Carrión VG. Mixed lateral preference in posttraumatic stress disorder. The Journal of nervous and mental disease 2006;194:142–4.
[41] Drake AI, Hannay HJ, Gam J. Effects of chronic Alcoholism on hemispheric functioning: An examination of gender differences for cognitive and dichotic listening tasks. Journal of clinical and experimental neuropsychology 1990;12:781–97.
[42] Alperin BR, Smith CJ, Gustafsson HC, Figuracion MT, Karalunas SL. The relationship between alpha asymmetry and ADHD depends on negative affect level and parenting practices. Journal of psychiatric research 2019;116:138–46.
[43] Dragovic M, Hammond G. Handedness in schizophrenia: a quantitative review of evidence. Acta psychiatrica Scandinavica 2005;111:410–9.
[44] Gordon E, Palmer DM, Cooper N. EEG alpha asymmetry in schizophrenia, depression, PTSD, panic disorder, ADHD and conduct disorder. Clinical EEG and neuroscience 2010;41:178–83.
[45] Thibodeau R, Jorgensen RS, Kim S. Depression, anxiety, and resting frontal EEG asymmetry: a meta-analytic review. Journal of abnormal psychology 2006;115:715–29.
[46] Davidson RJ. Anterior cerebral asymmetry and the nature of emotion. Brain and Cognition 1992;20:125–51.
[47] Hirnstein M, Hugdahl K. Excess of non-right-handedness in schizophrenia: meta-analysis of gender effects and potential biases in handedness assessment. The British journal of psychiatry the journal of mental science 2014;205:260–7.
[48] Logue DD, Logue RT, Kaufmann WE, Belcher HME. Psychiatric disorders and left-handedness in children living in an urban environment. Laterality 2015;20:249–56.
[49] Silberman EK, Weingartner H. Hemispheric lateralization of functions related to emotion. Brain and Cognition 1986;5:322–53.
[50] Rutherford HJV, Lindell AK. Thriving and Surviving: Approach and Avoidance Motivation and Lateralization. Emotion Review 2011;3:333–43.
[51] Leliveld LMC, Langbein J, Puppe B. The emergence of emotional lateralization: Evidence in non-human vertebrates and implications for farm animals. Applied Animal Behaviour Science 2013;145:1–14.
[52] Evans CS, Evans L, Marler P. On the meaning of alarm calls: functional reference in an avian vocal system. Animal Behaviour 1993;46:23–38.
[53] Dharmaretnam M, Rogers LJ. Hemispheric specialization and dual processing in strongly versus weakly lateralized chicks. Behavioural brain research 2005;162:62–70.
[54] Martín J, López P, Bonati B, Csermely D. Lateralization When Monitoring Predators in the Wild: A Left Eye Control in the Common Wall Lizard (Podarcis muralis). Ethology 2010;116:1226–33.
[55] Bonati B, Csermely D, López P, Martín J. Lateralization in the escape behaviour of the common wall lizard (Podarcis muralis). Behavioural brain research 2010;207:1–6.

[56] Robins A, Phillips C. Lateralised visual processing in domestic cattle herds responding to novel and familiar stimuli. Laterality 2010;15:514–34.
[57] Quaranta A, Siniscalchi M, Vallortigara G. Asymmetric tail-wagging responses by dogs to different emotive stimuli. Current biology CB 2007;17:R199-201.
[58] Filgueiras CC, Abreu-Villaça Y, Krahe TE, Manhães AC. Unilateral hemispherectomy at adulthood asymmetrically affects immobile behavior of male Swiss mice. Behavioural brain research 2006;172:33–8.
[59] Sullivan RM, Gratton A. Lateralized Effects of Medial Prefrontal Cortex Lesions on Neuroendocrine and Autonomic Stress Responses in Rats. The Journal of Neuroscience 1999;19:2834–40.
[60] Wittling W. The right hemisphere and the human stress response. Acta physiologica Scandinavica. Supplementum 1997;640:55–9.
[61] Richardson JS. On the functions of monoamine oxidase, the emotions, and adaptation to stress. The International journal of neuroscience 1993;70:75–84.
[62] Jones A, Osmond C, Godfrey KM, Phillips DIW. Evidence for developmental programming of cerebral laterality in humans. PloS one 2011;6:e17071.
[63] Brüne M, Nadolny N, Güntürkün O, Wolf OT. Stress induces a functional asymmetry in an emotional attention task. Cognition & emotion 2013;27:558–66.
[64] Moran JK, Crombach A, Elbert T, Nandi C, Bambonyé M, Wienbruch C et al. The individual contribution of DSM 5 symptom clusters of PTSD, life events, and childhood adversity to frontal oscillatory brain asymmetry in a large sample of active combatants. Biological psychology 2017;129:305–13.
[65] Dolensek N, Gehrlach DA, Klein AS, Gogolla N. Facial expressions of emotion states and their neuronal correlates in mice. Science (New York, N.Y.) 2020;368:89–94.
[66] Hausmann M, Güntürkün O. Steroid fluctuations modify functional cerebral asymmetries: the hypothesis of progesterone-mediated interhemispheric decoupling. Neuropsychologia 2000;38:1362–74.
[67] Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nature Reviews Neuroscience 2009;10:434–45.
[68] Kapoor A, Dunn E, Kostaki A, Andrews MH, Matthews SG. Fetal programming of hypothalamo-pituitary-adrenal function: prenatal stress and glucocorticoids. J Physiol 2006;572:31–44.
[69] Jacobson L. Hypothalamic-pituitary-adrenocortical axis: neuropsychiatric aspects. Comprehensive Physiology 2014;4:715–38.
[70] Schmitz J, Metz GAS, Güntürkün O, Ocklenburg S. Beyond the genome—Towards an epigenetic understanding of handedness ontogenesis. Progress in Neurobiology 2017;159:69–89.
[71] Carr CP, Martins CMS, Stingel AM, Lemgruber VB, Juruena MF. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes 201. United States; 2013 Dec.
[72] Neugebauer JM, Yost HJ. FGF signaling is required for brain left-right asymmetry and brain midline formation. Developmental biology 2014;386:123–34.
[73] Concha ML, Burdine RD, Russell C, Schier AF, Wilson SW. A Nodal Signaling Pathway Regulates the Laterality of Neuroanatomical Asymmetries in the Zebrafish Forebrain. Neuron 2000;28:399–409.

Morc1 as a potential new target gene in mood regulation: When and where to find in the brain

Annakarina Mundorf [a*], Jennifer Koch [a], Nadja Kubitza [a], Michaela Schmidt [b], Peter Gass [b], Nadja Freund [a]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany

[b] Department of Psychiatry and Psychotherapy, Central Institute of Mental Health Mannheim (ZI), Medical Faculty of Mannheim, University of Heidelberg, Mannheim, Germany

*Corresponding author

Research article / Manuscript in preparation

Abstract

Recent studies connected the *Morc family CW-type zinc finger 1* (*Morc1*) gene with early life stress and depression. Moreover, the *Morc* superfamily is related to epigenetic regulation in diverse nuclear processes. So far, *Morc1* was mainly studied in spermatogenesis whereas its distribution and function in the brain are still unknown. In a first attempt to characterize *Morc1* in the brain, its presence during development was investigated by Western Blot analysis. Then, *Morc1* realtime PCR was performed in different mood regulation brain areas in adult rats. MORC1 protein is already expressed in the brain at embryonic day 14 and stable expressed until adulthood. *Morc1* mRNA is present in many important brain areas of male rats. Areas like the dorsolateral and medial prefrontal cortex, the nucleus accumbens, the hippocampus, the hypothalamus, and the amygdala express *Morc1*. The wide distribution in the brain and its molecular structure as a zinc finger protein indicate that *Morc1* acts as a transcription factor. This function and its expression in mood regulating areas already in early development turn *Morc1* into a possible candidate gene for mediating early life stress and depression.

Keywords Development, early life stress, rat, depression, mood regulation

1. Introduction

For a long time, *MORC family CW-type zinc finger 1* (*MORC1*) was only known for its function in mammalian spermatogenesis (Inoue et al., 1999) where a *MORC1* deficiency leads to male-specific germ cell loss and infertility in mice (Pastor et al., 2014). Whereas a rat RNA BodyMap scan detected *MORC1* RNA in multiple organs, e.g. the brain, testes, and the thymus, with the highest amount of *MORC1* RNA found in the rat thymus (Yu et al., 2014). Recent studies connected *MORC1* to ELS and depression (Mundorf et al., 2018; Nieratschker et al., 2014; Schmidt et al., 2016; Thomas et al., 2020). However, until now, no study characterized the existence of *Morc1* in the brain. But the recent evidence of *MORC1* playing an important role in the brain and the development of psychiatric disorders asks for a further characterization.

In general, the early childhood is known to be a period of neuroplasticity and maturation of the brain (Johnston, 2004). Meanwhile, numerous studies confirm that interruption in neurodevelopment can have severe and long-lasting consequences (Teicher, 2002) while interruptions can be caused by multiple factors, one of which is early life stress (ELS).

It is well known that ELS can induce severe physical and mental changes lasting until or even first occurring in the adult life of affected persons (Carr et al., 2013). Traumatic or uncontrollable stressful events in early childhood, like sexual or physical abuse, loss of a caregiver or a natural disaster, are associated with the development of depression during adolescence (Andersen and Teicher, 2008; Kendler et al., 2004; Putnam, 2003). Even neuroanatomical changes as a result of ELS with changes mostly resulting in a volume reduction, of e.g. the hippocampus, the amygdala, the prefrontal cortex (PFC), and the corpus callosum (Frodl and O'Keane, 2013; Hart and Rubia, 2012) have been reported. Neuroanatomical changes subsequently lead to deficits in behavior, memory, and emotional perception (Hart and Rubia, 2012). In addition, ELS is affecting the activity of certain genes by altering DNA methylation. The altered methylation state of specific genes after ELS is known to influence the gene expression pattern (Labonté et al., 2012; Nieratschker et al., 2014; Weaver et al., 2004). Therefore, it seems that early life maltreatment sets the foundation for later structural changes in the brain (Frodl and O'Keane, 2013). Moreover, ELS is handled as a major high-risk factor for developing depression (Agid et al., 1999; Andersen and Teicher, 2008).

In a cross-species study, Nieratschker and colleagues found altered methylation of *MORC1* in children`s cord blood and in the brains of male rats after exposure to prenatal stress as well as in the blood of male nonhuman primates after ELS (Nieratschker et al., 2014). Moreover, they discovered that certain genetic variants of *MORC1* are connected to major depressive disorder (MDD). In a subsequent study, we were able to validate the depressive-like

behavior of female *MORC1* knockout mice (Schmidt et al., 2016). Recently, we could reinforce this link between *MORC1* methylation and depression as we discovered that *MORC1* hypermethylation was correlated to the Beck Depression Inventory (BDI) in healthy humans (Mundorf et al., 2018). In a multicentric study investigating childhood maltreatment, depressive symptoms and *MORC1* methylation, no association was found between childhood maltreatment and *MORC1* methylation but the association between *MORC1* methylation and depressive symptoms could be confirmed in all three cohorts (Thomas et al., 2020).

So far, MORC1 protein has already been characterized to have a CW-zing finger protein domain (Perry and Zhao, 2003). This protein domain is related to chromatin methylation status or early embryonic development (Perry and Zhao, 2003), reinforcing the important role of *MORC1* in development. Combined with the recent connection between ELS and altered methylation, it seems compelling to further investigate the expression pattern of *Morc1* in the rodent brain.

2. Material and Methods

2.1. Animals

Animals were group housed on a standard light-dark circle (12/12 hrs, light period 07:00–19:00) with water and food access *ad libitum* and constant temperature and humidity conditions (22 ± 2°C and 55 ± 25 %). Western blot was performed with the brains of male Spraque Dawley rats (Charles River Laboratories, Sulzfeld, Germany) at the age of postnatal day 2 (2 animals), postnatal day 22 (2 animals), and postnatal day 42 (2 animals). Western blotting was further carried out with the brain of one adult male and one adult female C57BL/6N as well as with one adult male and female C57BL/6N mouse with a Morc1−/− loss ($Morc^{Tg(Tyr)1Az/J}$, own breeding originally from the Jackson Labs (Bar Harbour, Maine, USA). Three adult (>postnatal day 60) male Spraque Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were included for realtime (rt) PCR analysis. All experiments were performed according to the principles regarding the care and use of animals adopted by the German Animal Welfare Law for the prevention of cruelty to animals after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Northrhine-Westfalia).

2.2. Western blot

All samples were homogenized, and protein was extracted with a Protease Inhibitor (Protease Inhibitor Cocktail Tablets cOmplete from Roche, 1 tablet diluted in 2 ml distilled H_20) and radioimmunoprecipitation assay buffer. An 8% SDS-Polyacrylamide gel was produced (ingredients from Roth). As marker Color Presteined Protein Standard (P7712S, New

England Biolabs) was used (245-11 kDa). Previous experiments revealed that for brain samples, 100 µg of protein were necessary for successful anti-MORC1 staining whereas 20µg protein of testes was sufficient. All samples were loaded with protein mixture and 5µl Lämmli Buffer on a 8% SDS-Polyacrylamide gel first at 100 V for 20 minutes, then for 1 $^{1}/_{2}$ h at 120V (BIO-RAD Power Supply). The protein was transferred from the gel to a Methanol activated PVDF-Membrane (0.45µm, Carl Roth GmbH) for 1 h at 100 V on ice. Afterwards, the membrane was blocked for one hour with gentle agitation in 5% non-fatty milk in 1x TBST at room temperature. Then, the membrane was incubated for one night at 4°C with the primary antibody anti-MORC1 from rabbit (1:500; 14080-1-AP, Proteintech). On the next day, after washing, the secondary antibody anti-rabbit HPR from goat (1:5000, 65-6120, Invitrogen) was incubated in 2% non-fatty milk in 1x TBST for one hour. After stripping, blots were incubated over night with anti-beta-Actin from rabbit (1:1500, 4967S, Cell Signaling) with gentle agitation in 0.5% BSA in 1x TBST. Second antibody was incubated in 0.5% BSA in 1x TBST for one hour. For chemiluminescent detection, the membrane was incubated with the enhanced chemiluminescent Thermo Scientific™ SuperSignal™ West Pico PLUS kit (1:1, 34087, Thermo Fisher). Blot imaging was then performed using the ChemiDoc™ MP Imaging System (BIO-RAD).

2.3. Realtime PCR

For rtPCR quantification of *Morc1* RNA brain regions were dissected from three adult male rats. Areas such as the medial (m) and dorsolateral (dl) PFC, nucleus accumbens (NAc), hippocampus, amygdala and hypothalamus were dissected according to the rat brain atlas (Paxinos and Watson, 2006) from three adult male rats. RNA isolation was performed using the NucleoSpin® TriPrep Kit (Macherey-Nagel, Düren, Germany) with slight modifications. For each sample, 40 µl of RNase-free water was added to obtain RNA. After quanitifcation and quality assessment, RNA was reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scientific, Darmstadt, Germany) according to the manufacturer's protocol. Experiments for establishing *Morc1* rtPCR revealed a minimum of 60 ng RNA needed per sample to detect stable results. Thus, 60 ng cDNA were used to measure *Morc1* mRNA levels in all regions. For rtPCR analysis, TaqMan™ Gene Expression Master Mix (Thermofisher) and TaqMan gene expression assay for Morc1 (Rn01474745_m1) as well as for Glyceraldehyde-3-phosphate dehydrogenase (Rn01775763_g1) and Actin, beta (Rn00667869_m1) serving as housekeeping genes were used. The 7500 Fast Real-Time PCR System (Applied Biosystems) was used according to manufacturer's protocol. RNA levels were

quantified by the number of cycle threshold (Delta CT method). All samples and gene assays were assayed in duplicates.

3. Results

3.1. Western blot

The rat brain samples at all age stages (E14, P2, P22, P41 and P>60 (Fig. 1A+B) revealed a prominent band at 110 kDa (Fig 1A). Interestingly, a light band at 110 kDa was also detected in the knockout mouse brain sample (Fig. 1C). However, when the intensity of the band was normalized with the beta-actin control (Fig. 1D) and then compared to the wildtype sample, the intensity for the knock-out was about 50% of the wildtype (females: 49%).

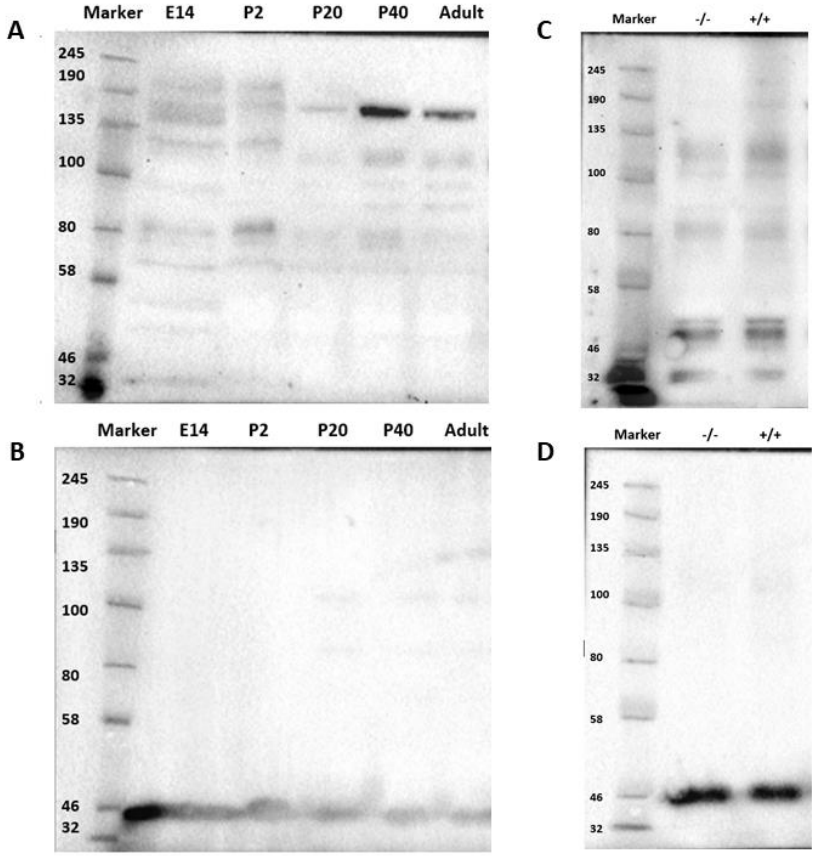


Figure 1: Western Blot imaging of **A** MORC1 protein (anti-MORC1 from rabbit, 1:500, 14080-1-AP, Proteintech) and **B** anti-beta-Actin from rabbit (1:1500, 4967S, Cell Signaling) in rat brain protein at the age of E14, P2, P22, P41 and adult (P>60). **C** MORC1 protein and **D** anti-beta-Actin imaging of one adult female C57BL/6N MORC1 knockout mouse (-/-) and in wildtype (+/+). Imaging was performed with the ChemiDoc™ MP Imaging System (BIO RAD). A prominent band was detected at 110 kDa.

3.2. Realtime PCR

Morc1 mRNA was detected in the mPFC (*M* = 16.46, *SD* = .7), dlPFC (*M* = 15.43, *SD* = .65), NAc (*M* = 12.84, *SD* = .62), hippocampus (*M* = 17.21, *SD* = .51), amygdala (*M* = 18.03, *SD* = .82) and hypothalamus (*M* = 16.87.46, *SD* = .29). Higher ΔCT value indicates less mRNA.

4. Discussion

Western blotting of the mouse and rat tissue detected prominent bands at 110 kDa which correspond to the estimated molecular weight of *Rattus norvegicus* MORC1 protein of 109 kDa (NCBI, 2016). Less protein was needed for detecting MORC1 in rat testis than for brain samples as testis is known to contain a high amount of *MORC1* RNA (Yu et al., 2014). This finding is in line with the high amount (60 ng) of RNA needed for rt PCR analysis of *Morc1*in the brain.

However, finding a promising antibody for anti-MORC1 staining proved to be difficult. As the chosen antibody is polyclonal, multiple bands besides the one at 110kDa were detected. Thus, investigating MORC1 protein expression in the brain, even though it is very important to analyze, would not have been accurate. Interestingly, western blotting against MORC1 protein displayed a slight band at 110 kDa in the knockout mouse brain, too. Given the properties of the knockout construct, this might not be surprising. For the knockout of MORC1, the Exons 2-4 were deleted and replaced by a sequence of almost the same length in the 3` region of MORC1 cDNA which leads to an aberrant mRNA transcript (Inoue et al., 1999). Thereby, it is possible that the knockout mouse expresses a shortened protein which in turn is not functional as it would be lacking the first residues of the MORC N-terminal region essential for functioning (Inoue et al., 1999). However, Pastor and colleagues (2014) did not detect MORC1 protein in the knockout mouse testis using immunohistochemistry (Pastor et al., 2014). But the used antibody was self-constructed and was thus not available for replication. Our chosen antibody might bind to a different side of the protein and therefore detect the truncated, unfunctional protein of the knockout. Moreover, western blotting might be more sensitive than immunohistochemistry as much more total protein is used for detection. The detected band in the knockout mouse was less intense than the wildtype mouse band. The knockout mouse might, therefore, express a low amount of an unfunctional MORC1 protein.

Detecting *Morc1* RNA in the amygdala, and the hippocampus suggests a role of *Morc1* in mood regulation as these regions are part of the limbic system (Borsook et al., 2016). Interestingly, parts of the mesolimbic dopaminergic pathway, like the NAc, the amygdala, and the hypothalamus further express *Morc1* RNA. These structures are part of the reward circuitry whose function is impaired in MDD (Dichter et al., 2012).

In general, ELS can lead to altered methylation state of genes (Labonté et al., 2012; Nieratschker et al., 2014; Weaver et al., 2004), including *MORC1* (Mundorf et al., 2018;

Nieratschker et al., 2014), and thereby affect gene expression. So far, it is known that the absence of MORC1 protein leads to depressive-like behavior (Schmidt et al., 2016) and certain genetic variants of *MORC1* are connected to MDD (Nieratschker et al., 2014). Recently, we could report an association between the hypermethylation of *MORC1* and higher BDI scores, as well as hints of a changed methylation after ELS (Mundorf et al., 2018). All these findings reinforce the important role *MORC1* plays during development and in the development of depression.

Based on the performed western blotting including different developmental stages of the brain, it was possible to detect MORC1 protein expression at all different ages. Given the fact, that MORC1 is expressed in early embryonic development, as well as in the entire brain, the gene may take an important part in the early development of the brain. As the expression does not seem to differ during development, an altered MORC1 expression induced by experienced ELS could be the trigger for structural changes. Impaired signal processing in specific brain regions could then lead to severe dysfunctions and structural changes, like the reduced volume of the hippocampus, amygdala, PFC and corpus callosum found after ELS (Frodl and O'Keane, 2013; Hart and Rubia, 2012).

It is already well known that dysfunction of transcriptional genes leads to impaired plasticity and thereby to severe disorders (Johnston, 2004) and cognitive impairment (Johnston et al., 2003). Given these facts, it does not seem too far-fetched to assume that *MORC1* as a transcription factor might be involved in the development of severe disorders like MDD.

There are already several studies linking ELS with depressive-like behavior and dysfunctional brain networks, but the pathogenesis is still unclear.

A study in rat pups discovered that ELS results in a dysfunction of prefrontal and mesolimbic regions in the young rat brain, which may contribute to behavioral changes later in life (Spivey et al., 2011). The mesolimbic system is one region where *Morc1* was found in our study, so altered *Morc1* expression after ELS in mesolimbic structures could be one reason for behavioral changes. Further, in male rats, ELS disrupts dendritic morphology of neurons in PFC, hippocampus, and NAc (Monroy et al., 2010). A disrupted function in any of these regions leads to MDD symptoms. Altered expression of *Morc1*, specifically given its role as a transcription factor, could be the reason for this dysmorphology.

Taken together, *Morc1* seems to be present in many important areas whose dysfunction can lead to the clinical picture of MDD. The wide distribution in the brain and its molecular structure indicate that *Morc1* acts as a transcription factor in the brain as well. Therewith, *Morc1* would be able to influence gene expression patterns within pathways involved in mood

regulation and emotion and might be the decisive structure that causes MDD after exposure to ELS. As high values of total protein and mRNA were needed for detecting *Morc1* expression, no region-specific protein expression pattern could be analyzed during development. Moreover, only regions providing at least 60 ng of total RNA were included for rt PCR analysis. Thus, more studies investigating different brain regions are needed to support *Morc1*s role in the brain.

Given that MORC1 protein already exists in early embryonic stages, ELS might influence its expression pattern right from the beginning and thereby impair regular brain development. Besides recent human studies investigating *MORC1* which significantly increased its connection to mood disorders, its functional role in the brain is still unknown. Therefore, more studies investigating the *Morc1* gene in the brain are highly needed.

Acknowledgments

We would like to thank Marie-Pierre Manitz and Felix Ströckens for their constant, fast and supportive advice regarding the methods and Vanessa Nieratschker for fruitful discussions.

Conflict of Interest

The authors declare no conflict of interest.

Author Contribution

A.M. designed the study, performed the experiments, and wrote the manuscript. N.F. designed the study and wrote the manuscript. P.G. and M.S. provided the knockout mouse. J.P. performed the western blots. N.K. helped with the experiments. All authors read and approved the manuscript.

Abbreviations

ELS: Early life stress

dlPFC: Dorsolateral prefrontal cortex

PFC: Prefrontal cortex

MDD: Major Depressive Disorder

mPFC: Medial prefrontal cortex

MORC1: MORC family CW-type zinc finger 1

NAc: Nucleus Accumbens

Rt: Realtime

References

Agid, O., Shapira, B., Zislin, J., Ritsner, M., Hanin, B., Murad, H., Troudart, T., Bloch, M., Heresco-Levy, U., Lerer, B., 1999. Environment and vulnerability to major psychiatric

illness: a case control study of early parental loss in major depression, bipolar disorder and schizophrenia. Mol. Psychiatry 4, 163–172.

Andersen, S.L., Teicher, M.H., 2008. Stress, sensitive periods and maturational events in adolescent depression. Trends Neurosci. 31, 183–191. https://doi.org/10.1016/j.tins.2008.01.004

Borsook, D., Linnman, C., Faria, V., Strassman, A.M., Becerra, L., Elman, I., 2016. Reward deficiency and anti-reward in pain chronification. Neurosci. Biobehav. Rev. 68, 282–297. https://doi.org/10.1016/j.neubiorev.2016.05.033

Carr, C.P., Martins, C.M.S., Stingel, A.M., Lemgruber, V.B., Juruena, M.F., 2013. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. J. Nerv. Ment. Dis. 201, 1007–1020. https://doi.org/10.1097/NMD.0000000000000049

Dichter, G.S., Damiano, C.A., Allen, J.A., 2012. Reward circuitry dysfunction in psychiatric and neurodevelopmental disorders and genetic syndromes: animal models and clinical findings. J. Neurodev. Disord. 4, 19. https://doi.org/10.1186/1866-1955-4-19

Frodl, T., O'Keane, V., 2013. How does the brain deal with cumulative stress? A review with focus on developmental stress, HPA axis function and hippocampal structure in humans. Neurobiol. Dis. 52, 24–37. https://doi.org/10.1016/j.nbd.2012.03.012

Hart, H., Rubia, K., 2012. Neuroimaging of child abuse: a critical review. Front. Hum. Neurosci. 6. https://doi.org/10.3389/fnhum.2012.00052

Inoue, N., Hess, K.D., Moreadith, R.W., Richardson, L.L., Handel, M.A., Watson, M., Zinn, A.R., 1999. New Gene Family Defined by MORC, a Nuclear Protein Required for Mouse Spermatogenesis. Hum. Mol. Genet. 8, 1201–1207. https://doi.org/10.1093/hmg/8.7.1201

Johnston, M.V., 2004. Clinical disorders of brain plasticity. Brain Dev. 26, 73–80. https://doi.org/10.1016/S0387-7604(03)00102-5

Johnston, M.V., Alemi, L., Harum, K.H., 2003. Learning, memory, and transcription factors. Pediatr. Res. 53, 369–374. https://doi.org/10.1203/01.PDR.0000049517.47493.E9

Kendler, K.S., Kuhn, J.W., Prescott, C.A., 2004. Childhood sexual abuse, stressful life events and risk for major depression in women. Psychol. Med. null, 1475–1482. https://doi.org/10.1017/S003329170400265X

Labonté, B., Suderman, M., Maussion, G., Navaro, L., Yerko, V., Mahar, I., Bureau, A., Mechawar, N., Szyf, M., Meaney, M.J., Turecki, G., 2012. Genome-wide epigenetic regulation by early-life trauma. Arch. Gen. Psychiatry 69, 722–731. https://doi.org/10.1001/archgenpsychiatry.2011.2287

Monroy, E., Hernández-Torres, E., Flores, G., 2010. Maternal separation disrupts dendritic morphology of neurons in prefrontal cortex, hippocampus, and nucleus accumbens in male rat offspring. J. Chem. Neuroanat. 40, 93–101. https://doi.org/10.1016/j.jchemneu.2010.05.005

Mundorf, A., Schmitz, J., Güntürkün, O., Freund, N., Ocklenburg, S., 2018. Methylation of MORC1: A possible biomarker for depression? J. Psychiatr. Res. 103, 208–211. https://doi.org/10.1016/j.jpsychires.2018.05.026

NCBI, 2016. MORC1 protein [WWW Document]. URL http://www.ncbi.nlm.nih.gov/protein/NP_001165561.1 (accessed 9.12.16).

Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nöthen, M.M., Suomi, S.J., Peus, V., Scharnholz, B., Dukal, H., Hohmeyer, C., Wolf, I. a.-C., Cirulli, F., Gass, P., Sütterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Transl. Psychiatry 4, e429. https://doi.org/10.1038/tp.2014.75

Pastor, W.A., Stroud, H., Nee, K., Liu, W., Pezic, D., Manakov, S., Lee, S.A., Moissiard, G., Zamudio, N., Bourc'his, D., Aravin, A.A., Clark, A.T., Jacobsen, S.E., 2014. MORC1 represses transposable elements in the mouse male germline. Nat. Commun. 5, 5795. https://doi.org/10.1038/ncomms6795

Paxinos, G., Watson, C., 2006. The Rat Brain in Stereotaxic Coordinates, 6th ed. Elsevier.

Perry, J., Zhao, Y., 2003. The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. Trends Biochem. Sci. 28, 576–580. https://doi.org/10.1016/j.tibs.2003.09.007

Putnam, F.W., 2003. Ten-Year Research Update Review: Child Sexual Abuse. J. Am. Acad. Child Adolesc. Psychiatry 42, 269–278. https://doi.org/10.1097/00004583-200303000-00006

Schmidt, M., Brandwein, C., Luoni, A., Sandrini, P., Calzoni, T., Deuschle, M., Cirulli, F., Riva, M.A., Gass, P., 2016. Morc1 knockout evokes a depression-like phenotype in mice. Behav. Brain Res. 296, 7–14. https://doi.org/10.1016/j.bbr.2015.08.005

Spivey, J.M., Padilla, E., Shumake, J.D., Gonzalez-Lima, F., 2011. Effects of maternal separation, early handling, and gonadal sex on regional metabolic capacity of the preweanling rat brain. Brain Res. 1367, 198–206. https://doi.org/10.1016/j.brainres.2010.10.038

Teicher, M.H., 2002. Scars that won't heal: the neurobiology of child abuse. Sci. Am. 286, 68–75.

Thomas, M., Coope, A., Falkenberg, C., Dunlop, B.W., Czamara, D., Provencal, N., Craighead, W.E., Mayberg, H.S., Nemeroff, C.B., Binder, E.B., Nieratschker, V., 2020. Investigation of MORC1 DNA methylation as biomarker of early life stress and depressive symptoms. J. Psychiatr. Res. 120, 154–162. https://doi.org/10.1016/j.jpsychires.2019.10.019

Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. https://doi.org/10.1038/nn1276

Yu, Y., Fuscoe, J.C., Zhao, C., Guo, C., Jia, M., Qing, T., Bannon, D.I., Lancashire, L., Bao, W., Du, T., Luo, H., Su, Z., Jones, W.D., Moland, C.L., Branham, W.S., Qian, F., Ning, B., Li, Y., Hong, H., Guo, L., Mei, N., Shi, T., Wang, K.Y., Wolfinger, R.D., Nikolsky, Y., Walker, S.J., Duerksen-Hughes, P., Mason, C.E., Tong, W., Thierry-Mieg, J., Thierry-Mieg, D., Shi, L., Wang, C., 2014. A rat RNA-Seq transcriptomic BodyMap across 11 organs and 4 developmental stages. Nat. Commun. 5, 3230. https://doi.org/10.1038/ncomms4230

An investigation of mPFC Morc1 RNA expression in juvenile, adolescent and adult rats after early stress exposure

Annakarina Mundorf *[1] and Nadja Freund [1]

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

*Corresponding author

Research article / in preparation

Abstract

Stress exposure during the early and late childhood causes long-lasting neurobiological and behavioral impairments. These alterations can change throughout life, indicating sensitive periods not only of exposure but also for phenotype manifestation. Implications of exposure thus become more pronounced during periods of brain maturation.

In this study, we examined the consequences of maternal separation (MS), social isolation (ISO), and both stressors consecutively on anxiety behavior and gene expression. Therefore, female and male rats were first exposed to either no, one, or both stressors and then tested for altered anxiety in juvenility, adolescence, or adulthood. Moreover, mRNA levels of *Morc1*, a target gene associated with ELS and depression, were measured in the medial prefrontal cortex allowing to investigate changes on mRNA level throughout life.

Early exposure results in less anxiety in juveniles, whereas exposure leads to increased anxiety in adolescence, with higher levels of anxiety after ISO or cumulative exposure. Neither exposure to MS, ISO nor both caused detectable alterations in adults. However, in adults, sex-specific alterations in anxiety were observed independent of exposure, with females being less anxious than males. No significant differences in *Morc1* mRNA expression were found between any group at any age.

This study reinforces the dynamic effects of early exposure throughout life underlining the critical period of adolescence and highlighting sex-specific stress susceptibility. Moreover, the results indicate that implications of exposure on *Morc1* mRNA expression might be narrowly restricted to a certain sensitive period.

Keywords: Maternal separation, social isolation, depression, anxiety, brain development, female, male, Sprague-Dawley, elevated plus maze, marble burying, rt-PCR

1. Introduction

Experiencing traumatic stress in early life is a high-risk factor for psychopathologies and can induce long-lasting neurobiological alterations (Carr et al., 2013; Teicher et al., 2016). Especially, the risk to develop an anxiety disorder early on is increased after early exposure (Carr et al., 2013). Interestingly, the median age of onset of anxiety disorders is one of the earliest among psychiatric disorders with a mean age of 11 years with half of all lifetime cases beginning at the age of 14 years (Kessler et al., 2005). Additionally, many patients experience a high transition from depression to anxiety and vice versa while entering adolescence (Costello et al., 2003). Besides the transition between disorders, comorbidity is also frequently found. Almost 60% of patients diagnosed with an anxiety disorder suffer from depression as well (Zimmerman et al., 2000). Having a psychiatric disorder beginning in childhood also increases the prevalence of having comorbid psychiatric disorders later in life (Bittner et al., 2007; Copeland et al., 2013). Also, psychiatric disorders like depression and anxiety are in the top ten leading causes for years lived with disability across 188 countries (Global Burden of Disease Study 2013 Collaborators, 2015) underlining them as an increasing global burden.

Early life stress (ELS) during sensitive periods of brain development and maturation furthermore leads to neuroanatomical changes, such as reduced volumes of the hippocampus, corpus callosum, amygdala, and the prefrontal cortex resulting in deficits in memory, emotional perception, and behavior (Hart and Rubia, 2012). The hippocampus, prefrontal cortex, and amygdala are crucial brain structures in this context as they are involved in regulating the hypothalamus-pituitary-adrenal (HPA) axis, experience prolonged maturation until childhood and adolescence and are thus most vulnerable to demonstrate permanent alterations after stress exposure (Lupien et al., 2009). Additionally, certain genetic risk variants and epigenetic modifications in DNA packaging and transcription are found to interact with ELS resulting in psychopathology (Aragam et al., 2011; Farrell and O'Keane, 2016; Sullivan et al., 2009), indicating that the etiology of psychiatric disorders is influenced by a gene x environment interaction. Thus, epigenetic modifications hold the potential to mediate between exposure and psychopathology (Barker et al., 2017; Cecil et al., 2020). It is proposed that stress induces alterations in the DNA methylation pattern of specific genes, leading to harmful gene silencing or overexpression causative for psychopathology.

The *MORC family CW-type zinc finger 1* (*MORC1*) gene has been connected to ELS and depression in multiple studies and thus seems to be a promising target gene in mediating exposure and pathology (Mundorf et al., 2018; Nieratschker et al., 2014; Thomas et al., 2020). Nieratschker and colleagues were the first to report an association between reduced *MORC1*

methylation and ELS in human umbilical cord blood, primate blood, and rat brain tissue. Also, they reported a link between single nucleotide polymorphisms in the *MORC1* gene and MDD using a genome-wide association study database (Nieratschker et al., 2014). The reported association of polymorphisms in the *MORC1* nucleotide sequences, possibly resulting in reduced or silenced gene expression, and MDD was confirmed with a gene knockout in mice leading to depressive-like behavior (Schmidt et al., 2016). To strengthen the finding of *MORC1* as a target gene for ELS and depression, we analyzed *MORC1* methylation patterns of buccal cells in healthy participants who conducted the Beck Depression Inventory (BDI), a questionnaire frequently used to assess depressive symptoms revealing increased *MORC1* methylation associated with increased depressive symptoms (Mundorf et al., 2018). Moreover, the participants were asked to report whether they had experienced any complications during their birth. Interestingly, higher numbers of complications during birth were associated with decreased *MORC1* methylation, matching the previously reported hypomethylation after ELS (Mundorf et al., 2018; Nieratschker et al., 2014). Furthermore, *MORC1* methylation patterns were investigated in a multicentric study, analyzing whole blood DNA methylation from depressed patients, and matched healthy controls. Again, participants were also asked to report a history of childhood trauma. However, no link between childhood trauma and altered *MORC1* methylation was found but, in all three cohorts, increased *MORC1* methylation correlated with symptoms of depression (Thomas et al., 2020).

Consequently, questions arise about mechanisms acting between the found hypomethylation after ELS and the hypermethylation linked to depression, whether the DNA modifications act independently of each other or if a shift in directionality throughout development occurs. Therefore, it needs to be investigated whether reversible processes during development are responsible for the contrariwise methylation pattern of *MORC1* or if different modifications are involved in exposure and psychopathology. To answer these questions, animal models can help as they allow for an environmentally controlled, longitudinal study design and molecular analysis of the brain.

An established model to induce ELS in animals is the maternal separation (MS) paradigm. In brief, the pups are separated from their mother and littermates for several hours daily during the first postnatal weeks (Lehmann and Feldon, 2000; Tractenberg et al., 2016). The induced neurobiological and behavioral alterations differ depending on the postnatal days and hours chosen for separation (Freund et al., 2013; Peña et al., 2017). MS in rats induces depressive-like behavior (Leussis et al., 2012), anxiety (Honeycutt et al., 2020), and increased reward-seeking behavior (Brenhouse et al., 2013). Moreover, MS leads to decreased neuronal plasticity

(Andersen and Teicher, 2004; Gildawie et al., 2020). However, MS is a pre-weaning stressor and is thus applicable until the pups are weaned. Therewith, MS induces stress only during early childhood. To test whether stress exposure during late childhood has a different impact on offspring development, social isolation (ISO) can be applied as a post-weaning stressor. For ISO, the animal is single housed over a distinct period of days or weeks, whereas the controls are housed in groups. ISO induces anxiety and depression-like phenotypes and alters neurobiology similar to MS (Mumtaz et al., 2018; Weiss et al., 2004). Both MS and ISO are paradigms where stress is mainly induced by social isolation and are thus frequently used alternately or consecutively, allowing for the investigation of different sensitive periods of exposure as well as of cumulative exposure (Biggio et al., 2014; Jaric et al., 2019; Lukkes et al., 2009; Vargas et al., 2016).

To investigate whether neurological alterations induced by ELS also result in an altered phenotype, this study observed behavior as well. As symptoms of psychopathologies are more difficult to assess in childhood and given the high transition rate between disorders, altered anxiety behavior was chosen as the phenotype to be investigated throughout development. Anxiety-behavior is commonly assessed using the elevated plus maze (EPM) and the marble burying (MB) test (de Brouwer et al., 2019; Pellow et al., 1985; Poling et al., 1981). The EPM consists of two open and two closed arms allowing the animal to avoid the anxiety-inducing open arms by staying in the closed arms (Pellow et al., 1985; Pellow and File, 1986). Whereas in the MB, the animal is placed in a cage with normal bedding and 20 unfamiliar marbles. As the marbles are considered to be an aversive stimulus, anxious animals tend to bury them deep into the bedding material (de Brouwer et al., 2019; Poling et al., 1981).

As mentioned in the beginning, the prefrontal cortex is one structure that develops over a long period and thus is vulnerable to disruptions during childhood and adolescence (Lupien et al., 2009). Moreover, the medial prefrontal cortex (mPFC) has already been repeatedly reported to be involved in the etiology of depression and anxiety (for review see (Chocyk et al., 2013) and was previously reported to show decreased *Morc1* expression after early exposure (Nieratschker et al., 2014). Given the crucial time of exposure and induced phenotype, ELS induced changes in gene expression might be most prominent in the mPFC.

This study investigates the consequences of ELS experienced during critical periods of brain development on altered gene expression and behavior. Therefore, ELS will be induced by MS and ISO. Then, animals will be tested for altered anxiety behavior during juvenility, adolescence, and adulthood. Behavioral testing is followed by measuring mPFC mRNA levels

of the target gene *Morc1* contemporaneously. Therewith, the consequences of ELS during developmental stages will be analyzed allowing to complement the role of *Morc1* as a mediator between exposure and psychopathology.

2. Methods

2.1 Animals

A total of 32 pregnant Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) were singly housed in standard Makrolon IV cages upon arrival between gestational days 13-15. Animals were housed under standard conditions (22± 2°C room temperature, 55 ± 25 % humidity) and standard lighting (12h/12h) with free access to water and food. Offspring was either group-housed with same-sex littermates or single housed. The day of birth was considered postnatal day (PND) 0. At PND2, all pups were sexed and culled to 10 per litter when necessary (if possible five females and five males). Rats were weaned at PND21. All experiments were conducted under the principles of Germany's Animal Welfare Act after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz North Rhine-Westphalia). Pregnant rats were randomly assigned to MS or control group (CG) by an independent party. Animals were tested in two cohorts. In cohort 1 (8 MS dams, 8 CG dams), offspring were behaviorally tested and sacrificed in juvenility and adolescence, respectively. In cohort 2 (8 MS dams, 8 CG dams), offspring were behaviorally tested and sacrificed in adulthood. Four pregnant dams (2 MS, 2 CG) and later their offspring were tested simultaneously. One day after behavioral testing, animals were deeply anesthetized with intraperitoneal ketamine and xylazine (ratio of 2:1) injection and sacrificed by decapitation. Brains were extracted and stored at -80°C until further use. Preparation of the mPFC was performed according to Paxinos and Watson's The Rat Brain in Stereotaxic Coordinates (Paxinos and Watson, 2006).

2.2 Stress exposure

MS was conducted as previously described (Leussis et al., 2012). In brief, pups were separated from dam and littermates for 4 hours daily over PND 2-20, during the dark (=active) phase. For separation, pups were placed in separate cages. Until the pups were able to heat themselves, they were kept on a heating mat adjusted to 37°C with home cage bedding, separated from littermates by a self-made plastic grid that allowed auditory and olfactory but no physical contact. CG pups were only separated from dams to be weighed every 4-5 days. On the day of weaning at PND21, one pup per sex and litter was tested for anxiety-like behavior. Again, one

animal per litter and sex was single housed until PND40, experiencing social isolation (ISO) as a late stressor. The other pups were group-housed with same-sex littermates until they reached adolescence (PND40). Animals from the ISO group were group-housed with same-sex ISO animals from PND41-60. Therewith, four groups derive (I) early stress exposure (MS), (II) late stress exposure (ISO), (III) early and late stress exposure (MSISO), and (IV) no stress exposure (control group, CG). Moreover, animals were behaviorally tested as juveniles, adolescents, and adults, respectively.

2.3 Anxiety-like behavior

Anxiety-like behavior was assessed using the EPM and the MB test. Briefly, in the EPM, animals were exposed to a maze with four arms (50 cm x 10 cm) 50 cm from the ground. Two arms were closed providing protection, and two opposing arms were open. Animals were placed in the maze always facing the same closed arm and were then observed for 5 minutes. The time spent in the open and closed arms respectively was manually scored. In the MB test, 20 marbles were placed symmetrically (4x5 rows) in a new standard Makrolon IV cage on smoothened bedding. After a 15-minutes trial, the number of marbles fully covered with bedding was counted. Juveniles were tested in the EPM followed by MB on the same day. Adolescents and adults were tested in the EPM followed by MB on the next day. Testing was always performed during the dark (= active) phase. All tests were rated by the same experimenter blinded to condition. The total number of animals per group and sex are given in table 1.

Table 1: Number of animals tested per age, group, and sex. MS: Maternal separation, CG: controls, ISO: social isolation, MSISO: MS + ISO.

Age tested	**MS**	**CG**	**ISO**	**MSISO**
Juvenile	8 ♀ + 8 ♂	8 ♀ + 8 ♂	-	-
Adolescent	23 ♀ + 22 ♂	24 ♀ + 24 ♂	8 ♀ + 8 ♂	8 ♀ + 8 ♂
Adult	31 ♀ + 31 ♂	32 ♀ + 31 ♂	8 ♀ + 8 ♂	8 ♀ + 8 ♂

2.4 Real-time PCR mRNA Analysis

RNA was extracted using the NucleoSpin® TriPrep (Macherey-Nagel, Düren, Germany) with slight modifications. Briefly, 40 µl of RNase-free water was added to each sample to obtain RNA. Then, concentration and quality of RNA were measured using the NanoDrop™ ND-1000 Spectrophotometer (PEQLAB Biotechnologie, Erlangen, Germany). RNA was converted to

cDNA using the High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scientific, Darmstadt, Germany). For real-time PCR analysis, the TaqMan™ Gene Expression Master Mix and primers were used. Three different TaqMan gene expression assays were used. *Morc1* (Rn01474745_m1) as target gene and *Glyceraldehyde-3-phosphate dehydrogenase* (Rn01775763_g1) and *Actin, beta* (Rn00667869_m1) as two housekeeping genes, respectively. A testis sample was used as a reference sample. All samples and genes were assayed in duplicates. Real-time PCR (Applied Biosystems 7500 Fast Real-Time PCR System) reaction was performed according to the manufacturer´s protocol. The number of cycle thresholds (delta CT method) was assessed for each gene. To exclude an effect of plate, delta-delta CT was calculated. Due to technical errors or insufficient ng of extracted mRNA, in total, seven juveniles, 18 adolescents, and 15 adults had to be excluded from the analysis.

2.3 Statistical analysis

Behavior and mRNA levels were analyzed for every age separately. Multivariate analysis of variance (MANOVA) was conducted with treatment and sex as factors and time spend on the open arms, number of marbles fully covered as dependent variables. mRNA levels were analyzed using ANOVA. Bonferroni correction was used for post hoc analysis. In juveniles, sex had no significant effect, therefore, a student's t-test was performed with treatment as factor and behavior as dependent variables and mRNA levels, respectively. To control for false discovery rate (FDR) when analyzing multiple comparisons significance was set at $p < .0125$ ($p = .05$ divided by 2 groups with 2 dependent variables). Outliers were identified by percentile analysis and Tukey's hinges meaning that values outside the 25 and 75 percentiles were excluded. Data were analyzed using SPSS (IBM SPSS Statistics 26).

3. Results

3.1 Juveniles

MS juveniles spent significantly more time in the open arms than CG juveniles $t(19.17) = -3.11$, $p = .006$ and buried fewer marbles in total compared to CG juveniles $t(27.55) = 2.159$, $p = .040$, (**Fig. 1**). In the MB, one MS male and in EPM testing, two MS males had to be excluded from analysis due to outlier status. Comparing *Morc1* delta-delta CT between groups showed no difference in mRNA expression $t(23) = -.76$, $p = .455$.

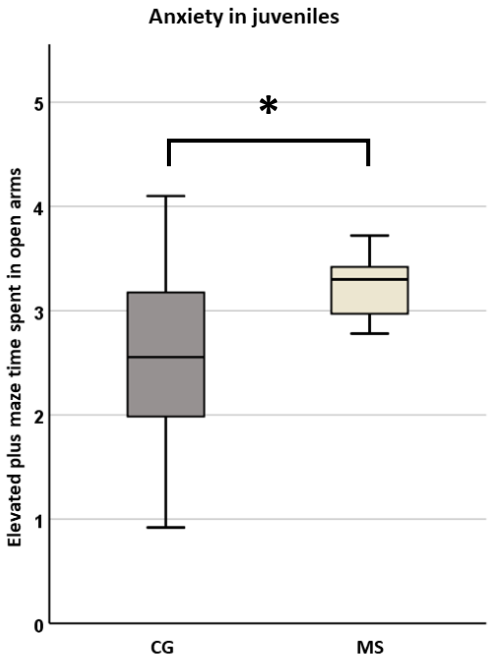


Figure 1: Anxiety behavior after stress exposure assessed in juveniles. The two different stress groups are shown. Dark gray: CG, light gray: MS. Time spent in the open arms in the EPM in minutes. MS juveniles spent significantly more time in the open arms (*p =.006).

3.2 Adolescents

No differences were found when comparing the time spent in the open arms in the EPM regardless of group F(3,112) = .932, p = .428 or sex F(1, 112) = 2.681, p = .104. But analyzing the number of marbles buried in the MB revealed a significant effect of exposure F(3,112) = 9.657, p = .000 and sex F(1, 112) = 5.547, p = .020. Post hoc analysis showed that CG buried significantly fewer marbles than ISO (p = .001) or MSISO (p = .000) and MS adolescents buried significantly fewer marbles than MSISO (p = .029). There was no difference in the number of marbles buried between ISO and MSISO (p = 1.00) (**Fig. 2**). For statistical analysis, one MSISO female and three males had to be excluded from EPM, and one female MSISO from MB.

Morc1 delta-delta CT means did not differ between groups F(3, 38) = .274, p = .844 or sex F(1, 38) = 1.42, p = .241. For the analysis of delta-delta CT levels, two female CG and one male CG had to be excluded due to outlier status.

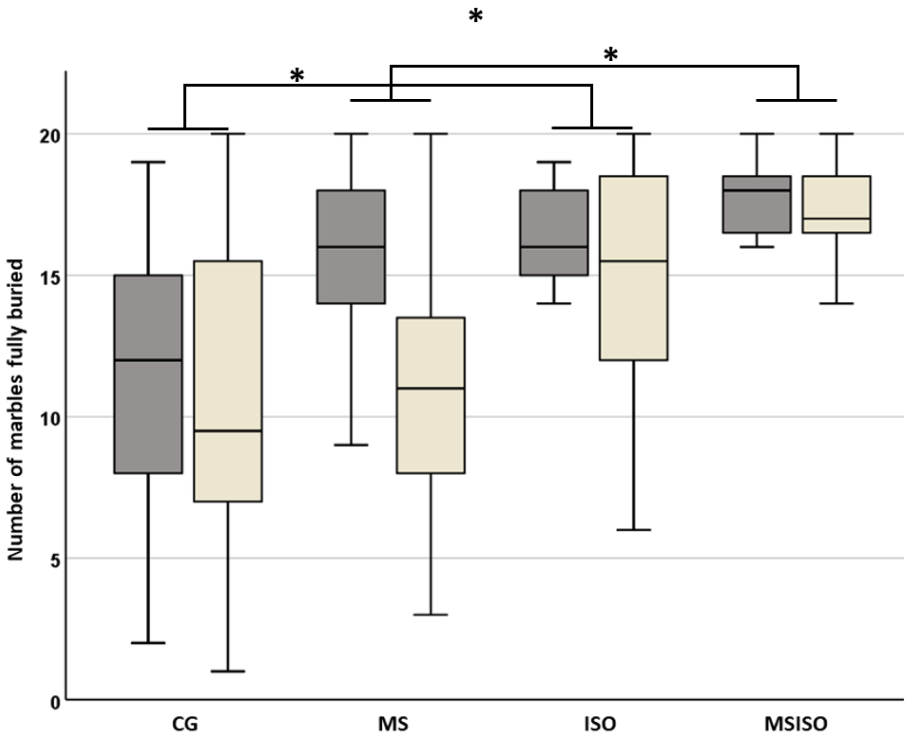


Figure 2: Anxiety behavior after stress exposure assessed in adolescents. The four different stress groups are shown. Dark gray: Males, light gray: females. Number of marbles fully covered. Adolescent CG buried significantly fewer marbles than ISO (p = .001) or MSISO (p = .000). MS adolescents buried significantly fewer marbles than MSISO (p = .029). * p < .03.

3.3 Adults

Comparing the time spent in the open arms in the EPM showed no effect of exposure F(3, 138) = 1.644, *p* = .182 but differences in anxiety depending on sex F(1, 138) = 14.912, *p* = .000 (**Fig. 3A**). In the MB, no effect of stress exposure was found F(3, 138) = .869, *p* = .459 but again an effect of sex F(1, 138) = 52.901, *p* = .000 (**Fig. 3B**).

ANOVA of *Morc1* delta-delta CT levels revealed no effect of sex F(1, 37) = 2.582, *p* = .117 but a trend towards an effect of exposure F(3, 37) = 2.763, *p* = .056. Interestingly, an interaction between sex*exposure was shown F(3, 37) = 3.016, *p* = .042. Due to outlier status, the following values had to be excluded: for EPM: five male and one female CG and one MS female; for MB: one ISO female; delta-delta CT: two MS females and two ISO males.

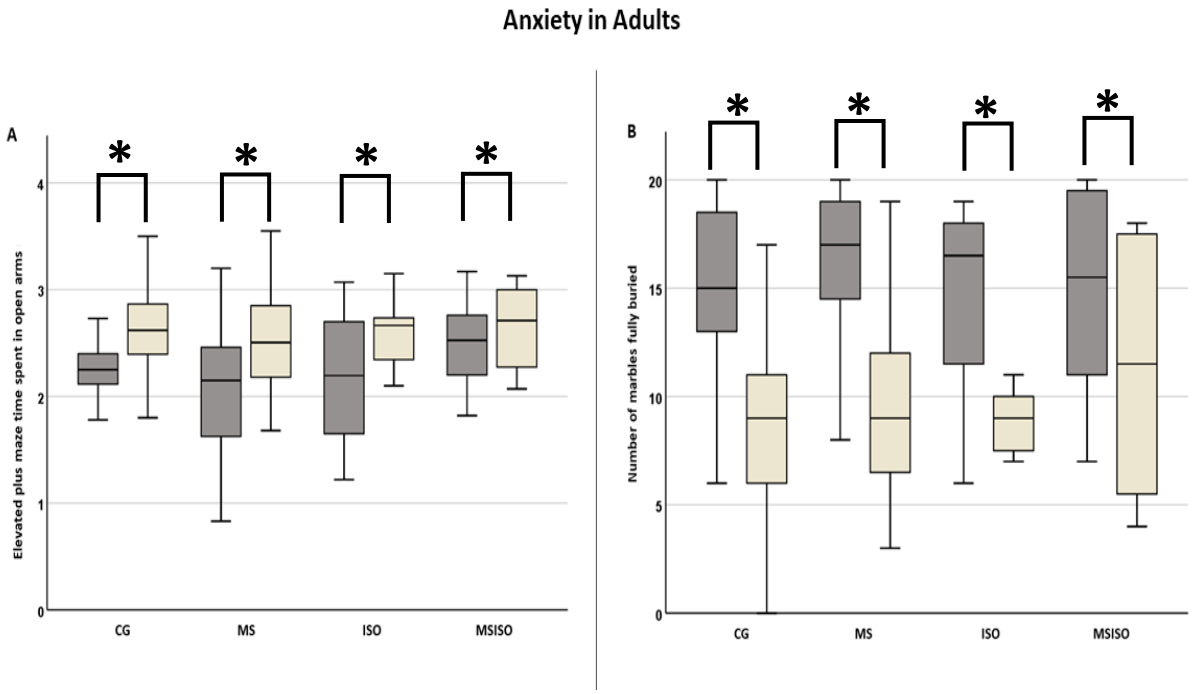


Figure 3: Anxiety behavior after stress exposure assessed in adults. The four different stress groups are shown. Dark gray: Males, light gray: females. **A**: Time spent in the open arms in the EPM in minutes. Adult females spent significantly more time in the open arms than males independent of exposure (p = .000). **B**: Number of marbles fully covered. Adult females buried significantly fewer marbles than males independent of exposure (p = .000). * p = .001

4. Discussion

In this study, the consequences of early stress exposure on anxiety behavior and *Morc1* mRNA levels in the mPFC in juvenile, adolescent, and adult rats were investigated. Animals were exposed to MS, ISO, MSISO, or no stress during childhood and tested in the EPM and MB afterward. Interestingly, different effects of exposure were found depending on the age of testing. Juveniles exposed to MS showed less anxiety. Testing for anxiety behavior in adolescents revealed that stress exposure in late childhood leads to increased anxiety in the MB, as CG animals buried significantly fewer marbles than ISO or MSISO rats. Interestingly, MS adolescents only buried significantly fewer marbles compared to MSISO adolescents. However, testing adults that were group-housed during adolescence after early exposure showed no effect of exposure on anxiety behavior. In both tests, adult females were less anxious than adult males, independently of exposure. Thus, the results indicate that the consequences of exposure on anxiety vary depending on the age of testing. Moreover, it shows that induced behavior is flexible and changing over time. The investigated mRNA expression of *Morc1* in the mPFC did not reveal any significant differences between groups.

The fact, that juveniles exposed to MS were less anxious than controls might be striking at first, considering the preceding chronic exposure. However, the daily experience of social isolation in a new environment during MS might render juveniles less anxious when tested one day after the last separation. In contrast, CG juveniles were only separated from the dam to be weighed, rendering them more anxious in new situations. Moreover, when investigating maternal behavior in MS dams, increased maternal care towards pups upon reunion is found compared to CG dams (Bölükbas et al., submitted), indicating a potential protective effect of increased maternal care against MS, especially, as high maternal care has a positive influence on the stress response (Weaver et al., 2004). Furthermore, our study underlines adolescence as a crucial time, as the effects of stress exposure on behavior were most severe at this age. Adolescence is a time of immense brain growth and maturation as important neuronal networks like the reward system, the amygdala-prefrontal cortex circuitry regulating negative affect, and the social brain are being matured and strengthened in this period (Blakemore, 2012; Galván, 2010; Tottenham and Galván, 2016). Thus, neurobiological alterations induced by exposure during childhood that result in altered behavior might be most pronounced in adolescence when distinct networks are being intensified. Since ELS is known to disrupt neuronal growth of e.g., the prefrontal cortex, hippocampus, and amygdala (Lupien et al., 2009), alterations in the development of one region alone might not be leading to changes in behavior but, considering the development of trajectories, reduced activity in one region does impact circuitry balances during maturation resulting in more profound disruptions. For example, ELS leads to an increased amygdala: hippocampus ratio (Andersen and Teicher, 2008), therewith altering the normal interregional communication. During the maturation of the amygdala-prefrontal cortex circuitry in adolescence, shifted activity between these two regions can then lead to symptoms of psychopathologies (Gee et al., 2013; Tottenham and Galván, 2016). Interestingly, MS does not seem to affect females, as they are as anxious as CG adolescents, whereas MS males show similar anxiety to ISO and MSISO adolescents in the MB. However, adult rats showed no behavioral implications after exposure. Given that all rats tested in adulthood were group-housed during adolescence, and as rats are social animals, group-housing might be a mediating factor against exposure. Therefore, group-housing after exposure might reverse behavioral effects found in adolescence. Interestingly, adult females that were previously exposed to MSISO seem to be more anxious than CG, MS, or ISO females (though not significantly), suggesting that the time spent in group-housing was not sufficiently long enough to reverse the alterations induced by both stressors. The sex difference found in adolescents and adults with females being less anxious than males is frequently being reported (Jaric et al., 2019; Kokras

and Dalla, 2014; Leussis et al., 2012). Especially in terms of MS, females might be more resilient to express phenotypic implications compared to males. This is in line with previous reports of MS-induced depression-like behavior only found in adult male rats but not females, indicating a potential resilience of females against MS (Dimatelis et al., 2016). Generally, sex-specific impairments of exposure on neurobiology and behavior have repeatedly been reported (Freund et al., 2013; Honeycutt et al., 2020; Jaric et al., 2019; Leussis et al., 2012; Mundorf et al., 2019; Schroeder et al., 2018) underlining the importance to include both sexes.

Studies combining MS and ISO stress frequently find different implications of cumulative exposure and single exposure, such as MS followed by ISO results in a weaker reduction of hippocampal plasticity compared to ISO alone whereas corticosterone levels are more reduced after experiencing both stressors compared to ISO (Biggio et al., 2014). Moreover, MS or ISO alone results in the greatest effect on anxiety- and depression-like behavior in adulthood compared to solely MS or ISO (Jaric et al., 2019). Interestingly, ISO following MS reduced the effects of the early stressor on behavior in adulthood, but the effects were mostly female-specific (Jaric et al., 2019). However, in this study, combining MS and ISO did not result in increased impairment or decreased impairment but rather increases anxiety behavior equally to MS or ISO alone.

The analysis of *Morc1* mRNA expression in the mPFC revealed no differences between groups at any age. However, Thomas et al. also reported no association between ELS and altered *Morc1* methylation in adults diagnosed with depression (Thomas et al., 2020). Opposing is the previously found hypomethylation in the mPFC of adult rats after prenatal stress exposure (Nieratschker et al., 2014) and after birth complications in humans (Mundorf et al., 2018). Therefore, the timing of exposure might be crucial, as the gene hypomethylation was found after pre- and perinatal exposure to stress (Mundorf et al., 2018; Nieratschker et al., 2014) implicating that the methylation pattern of *Morc1* may be most sensitive to stress-induced changes not during early postnatal days, but in fetal brain development. Similar indications of narrowly restricted sensitive periods have already been reported for other genes involved in implicating stress susceptibility (Peña et al., 2017). Solely exposure during distinct postnatal days led to altered gene expression causative for increased stress susceptibility in adulthood (Peña et al., 2017). In the study, a second stressor in adulthood was needed to manifest early priming in depression-like behavior. Interestingly, it was further revealed that a knockout of the target gene in early life increases susceptibility whereas a knockout in adulthood did not (Peña et al., 2017) indicating the importance of distinct sensitive periods in brain development. As altered DNA methylation is frequently associated with altered gene expression (Razin and

Cedar, 1991), our study indicates that postnatal exposure during childhood might not lead to long-lasting changes in mRNA expression levels. Further studies should thus investigate *Morc1* expression patterns after prenatal exposure. Also, previously identified changes in methylation patterns might not consequently result in altered gene expression, in this specific case.

ELS induced altered anxiety with changes in directionality across development. Interestingly, juveniles exposed to ELS were less anxious marking early childhood as a time of great adaptability. Meanwhile, adolescence is a time where implications of ELS are most pronounced in phenotypic alterations. Group-housing after ELS seems to reverse the effects of exposure. The sex difference found in adults reinforces the need for including females in all studies, as females tend to be more resilient to exposure. Furthermore, implications of exposure on *Morc1* mRNA expression in the mPFC might be narrowly restricted to a certain sensitive period, therefore, expression was not affected by ELS.

Acknowledgments

We thank the whole experimental and molecular psychiatry lab for support and fruitful discussions.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

A.M. and N.F. designed the study. A.M performed the experiments and analyzed the data. The manuscript was written by A.M. Both authors revised and approved the manuscript. This manuscript is our original work and it is submitted for first publication.

Abbreviations

BDI: Beck Depression Inventory
CG: Control group
ELS: Early life stress
EPM: Elevated plus maze
ISO: Social isolation
MB: Marble burying
mPFC: Medial prefrontal cortex
MDD: Major depressive disorder
MORC1: *MORC family CW-type zinc finger 1*
MS: Maternal separation
MSISO: Maternal separation + social isolation

References

Andersen, S.L., Teicher, M.H., 2008. Stress, sensitive periods and maturational events in adolescent depression. Trends Neurosci. 31, 183–191. https://doi.org/10.1016/j.tins.2008.01.004

Andersen, S.L., Teicher, M.H., 2004. Delayed effects of early stress on hippocampal development. Neuropsychopharmacol. Off. Publ. Am. Coll. Neuropsychopharmacol. 29, 1988–1993. https://doi.org/10.1038/sj.npp.1300528

Aragam, N., Wang, K.-S., Pan, Y., 2011. Genome-wide association analysis of gender differences in major depressive disorder in the Netherlands NESDA and NTR population-based samples. J. Affect. Disord. 133, 516–521. https://doi.org/10.1016/j.jad.2011.04.054

Barker, E.D., Walton, E., Cecil, C.A.M., 2017. Annual Research Review: DNA methylation as a mediator in the association between risk exposure and child and adolescent psychopathology. J. Child Psychol. Psychiatry. https://doi.org/10.1111/jcpp.12782

Biggio, F., Pisu, M.G., Garau, A., Boero, G., Locci, V., Mostallino, M.C., Olla, P., Utzeri, C., Serra, M., 2014. Maternal separation attenuates the effect of adolescent social isolation on HPA axis responsiveness in adult rats. Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol. 24, 1152–1161. https://doi.org/10.1016/j.euroneuro.2014.03.009

Blakemore, S.-J., 2012. Development of the social brain in adolescence. J. R. Soc. Med. 105, 111–116. https://doi.org/10.1258/jrsm.2011.110221

Brenhouse, H.C., Lukkes, J.L., Andersen, S.L., 2013. Early life adversity alters the developmental profiles of addiction-related prefrontal cortex circuitry. Brain Sci. 3, 143–158. https://doi.org/10.3390/brainsci3010143

Carr, C.P., Martins, C.M.S., Stingel, A.M., Lemgruber, V.B., Juruena, M.F., 2013. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. J. Nerv. Ment. Dis. 201, 1007–1020. https://doi.org/10.1097/NMD.0000000000000049

Cecil, C.A.M., Zhang, Y., Nolte, T., 2020. Childhood maltreatment and DNA methylation: A systematic review. Neurosci. Biobehav. Rev. 112, 392–409. https://doi.org/10.1016/j.neubiorev.2020.02.019

Chocyk, A., Majcher-Maślanka, I., Dudys, D., Przyborowska, A., Wędzony, K., 2013. Impact of early-life stress on the medial prefrontal cortex functions - a search for the pathomechanisms of anxiety and mood disorders. Pharmacol. Rep. PR 65, 1462–1470.

Costello, E.J., Mustillo, S., Erkanli, A., Keeler, G., Angold, A., 2003. Prevalence and development of psychiatric disorders in childhood and adolescence. Arch. Gen. Psychiatry 60, 837–844. https://doi.org/10.1001/archpsyc.60.8.837

de Brouwer, G., Fick, A., Harvey, B.H., Wolmarans, D.W., 2019. A critical inquiry into marble-burying as a preclinical screening paradigm of relevance for anxiety and obsessive-compulsive disorder: Mapping the way forward. Cogn. Affect. Behav. Neurosci. 19, 1–39. https://doi.org/10.3758/s13415-018-00653-4

Dimatelis, J.J., Vermeulen, I.M., Bugarith, K., Stein, D.J., Russell, V.A., 2016. Female rats are resistant to developing the depressive phenotype induced by maternal separation stress. Metab. Brain Dis. 31, 109–119. https://doi.org/10.1007/s11011-015-9723-8

Farrell, C., O'Keane, V., 2016. Epigenetics and the glucocorticoid receptor: A review of the implications in depression. Psychiatry Res. 242, 349–356. https://doi.org/10.1016/j.psychres.2016.06.022

Freund, N., Thompson, B.S., Denormandie, J., Vaccarro, K., Andersen, S.L., 2013. Windows of vulnerability: maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. Neuroscience 249, 88–97. https://doi.org/10.1016/j.neuroscience.2013.06.066

Galván, A., 2010. Adolescent Development of the Reward System. Front. Hum. Neurosci. 4. https://doi.org/10.3389/neuro.09.006.2010

Gee, D.G., Humphreys, K.L., Flannery, J., Goff, B., Telzer, E.H., Shapiro, M., Hare, T.A., Bookheimer, S.Y., Tottenham, N., 2013. A Developmental Shift from Positive to Negative Connectivity in Human Amygdala–Prefrontal Circuitry. J. Neurosci. 33, 4584–4593. https://doi.org/10.1523/JNEUROSCI.3446-12.2013

Gildawie, K.R., Honeycutt, J.A., Brenhouse, H.C., 2020. Region-specific Effects of Maternal Separation on Perineuronal Net and Parvalbumin-expressing Interneuron Formation in Male and Female Rats. Neuroscience 428, 23–37. https://doi.org/10.1016/j.neuroscience.2019.12.010

Hart, H., Rubia, K., 2012. Neuroimaging of child abuse: a critical review. Front. Hum. Neurosci. 6. https://doi.org/10.3389/fnhum.2012.00052

Honeycutt, J.A., Demaestri, C., Peterzell, S., Silveri, M.M., Cai, X., Kulkarni, P., Cunningham, M.G., Ferris, C.F., Brenhouse, H.C., 2020. Altered corticolimbic connectivity reveals sex-specific adolescent outcomes in a rat model of early life adversity. eLife 9. https://doi.org/10.7554/eLife.52651

Jaric, I., Rocks, D., Cham, H., Herchek, A., Kundakovic, M., 2019. Sex and Estrous Cycle Effects on Anxiety- and Depression-Related Phenotypes in a Two-Hit Developmental Stress Model. Front. Mol. Neurosci. 12, 74. https://doi.org/10.3389/fnmol.2019.00074

Kessler, R.C., Berglund, P., Demler, O., Jin, R., Merikangas, K.R., Walters, E.E., 2005. Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. Arch. Gen. Psychiatry 62, 593–602. https://doi.org/10.1001/archpsyc.62.6.593

Kokras, N., Dalla, C., 2014. Sex differences in animal models of psychiatric disorders. Br. J. Pharmacol. 171, 4595–4619. https://doi.org/10.1111/bph.12710

Lehmann, J., Feldon, J., 2000. Long-term biobehavioral effects of maternal separation in the rat: consistent or confusing? Rev. Neurosci. 11, 383–408.

Leussis, M.P., Freund, N., Brenhouse, H.C., Thompson, B.S., Andersen, S.L., 2012. Depressive-like behavior in adolescents after maternal separation: sex differences, controllability, and GABA. Dev. Neurosci. 34, 210–217. https://doi.org/10.1159/000339162

Lukkes, J.L., Mokin, M.V., Scholl, J.L., Forster, G.L., 2009. Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and altered hormonal stress responses. Horm. Behav. 55, 248–256. https://doi.org/10.1016/j.yhbeh.2008.10.014

Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim, C., 2009. Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nat. Rev. Neurosci. 10, 434–445. https://doi.org/10.1038/nrn2639

Mumtaz, F., Khan, M.I., Zubair, M., Dehpour, A.R., 2018. Neurobiology and consequences of social isolation stress in animal model—A comprehensive review. Biomed. Pharmacother. 105, 1205–1222. https://doi.org/10.1016/j.biopha.2018.05.086

Mundorf, A., Knorr, A., Mezö, C., Klein, C., Beyer, D.K., Fallgatter, A.J., Schwarz, M., Freund, N., 2019. Lithium and glutamine synthetase: Protective effects following stress. Psychiatry Res. 281, 112544. https://doi.org/10.1016/j.psychres.2019.112544

Mundorf, A., Schmitz, J., Güntürkün, O., Freund, N., Ocklenburg, S., 2018. Methylation of MORC1: A possible biomarker for depression? J. Psychiatr. Res. 103, 208–211. https://doi.org/10.1016/j.jpsychires.2018.05.026

Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nöthen, M.M., Suomi, S.J., Peus, V., Scharnholz, B., Dukal, H., Hohmeyer, C., Wolf, I. a.-C., Cirulli, F., Gass, P., Sütterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species

differential methylation in association with early life stress as well as genome-wide association with MDD. Transl. Psychiatry 4, e429. https://doi.org/10.1038/tp.2014.75

Paxinos, G., Watson, C., 2006. The Rat Brain in Stereotaxic Coordinates, 6th ed. Elsevier.

Pellow, S., Chopin, P., File, S.E., Briley, M., 1985. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. J. Neurosci. Methods. https://doi.org/10.1016/0165-0270(85)90031-7

Pellow, S., File, S.E., 1986. Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. Pharmacol. Biochem. Behav. https://doi.org/10.1016/0091-3057(86)90552-6

Peña, C.J., Kronman, H.G., Walker, D.M., Cates, H.M., Bagot, R.C., Purushothaman, I., Issler, O., Loh, Y.-H.E., Leong, T., Kiraly, D.D., Goodman, E., Neve, R.L., Shen, L., Nestler, E.J., 2017. Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. Science 356, 1185–1188. https://doi.org/10.1126/science.aan4491

Poling, A., Cleary, J., Monaghan, M., 1981. Burying by rats in response to aversive and nonaversive stimuli. J. Exp. Anal. Behav. 35, 31–44. https://doi.org/10.1901/jeab.1981.35-31

Razin, A., Cedar, H., 1991. DNA methylation and gene expression. Microbiol. Rev. 55, 451–458.

Schmidt, M., Brandwein, C., Luoni, A., Sandrini, P., Calzoni, T., Deuschle, M., Cirulli, F., Riva, M.A., Gass, P., 2016. Morc1 knockout evokes a depression-like phenotype in mice. Behav. Brain Res. 296, 7–14. https://doi.org/10.1016/j.bbr.2015.08.005

Schroeder, A., Notaras, M., Du, X., Hill, R.A., 2018. On the Developmental Timing of Stress: Delineating Sex-Specific Effects of Stress across Development on Adult Behavior. Brain Sci. 8. https://doi.org/10.3390/brainsci8070121

Sullivan, P.F., de Geus, E.J.C., Willemsen, G., James, M.R., Smit, J.H., Zandbelt, T., Arolt, V., Baune, B.T., Blackwood, D., Cichon, S., Coventry, W.L., Domschke, K., Farmer, A., Fava, M., Gordon, S.D., He, Q., Heath, A.C., Heutink, P., Holsboer, F., Hoogendijk, W.J., Hottenga, J.J., Hu, Y., Kohli, M., Lin, D., Lucae, S., Macintyre, D.J., Maier, W., McGhee, K.A., McGuffin, P., Montgomery, G.W., Muir, W.J., Nolen, W.A., Nöthen, M.M., Perlis, R.H., Pirlo, K., Posthuma, D., Rietschel, M., Rizzu, P., Schosser, A., Smit, A.B., Smoller, J.W., Tzeng, J.-Y., van Dyck, R., Verhage, M., Zitman, F.G., Martin, N.G., Wray, N.R., Boomsma, D.I., Penninx, B.W.J.H., 2009. Genome-wide association for major depressive disorder: a possible role for the presynaptic protein piccolo. Mol. Psychiatry 14, 359–375. https://doi.org/10.1038/mp.2008.125

Teicher, M.H., Samson, J.A., Anderson, C.M., Ohashi, K., 2016. The effects of childhood maltreatment on brain structure, function and connectivity. Nat. Rev. Neurosci. 17, 652–666. https://doi.org/10.1038/nrn.2016.111

Thomas, M., Coope, A., Falkenberg, C., Dunlop, B.W., Czamara, D., Provencal, N., Craighead, W.E., Mayberg, H.S., Nemeroff, C.B., Binder, E.B., Nieratschker, V., 2020. Investigation of MORC1 DNA methylation as biomarker of early life stress and depressive symptoms. J. Psychiatr. Res. 120, 154–162. https://doi.org/10.1016/j.jpsychires.2019.10.019

Tottenham, N., Galván, A., 2016. Stress and the Adolescent Brain: Amygdala-Prefrontal Cortex Circuitry and Ventral Striatum as Developmental Targets. Neurosci. Biobehav. Rev. 70, 217–227. https://doi.org/10.1016/j.neubiorev.2016.07.030

Tractenberg, S.G., Levandowski, M.L., de Azeredo, L.A., Orso, R., Roithmann, L.G., Hoffmann, E.S., Brenhouse, H., Grassi-Oliveira, R., 2016. An overview of maternal separation effects on behavioural outcomes in mice: Evidence from a four-stage methodological systematic review. Neurosci. Biobehav. Rev. 68, 489–503. https://doi.org/10.1016/j.neubiorev.2016.06.021

Vargas, J., Junco, M., Gomez, C., Lajud, N., 2016. Early Life Stress Increases Metabolic Risk, HPA Axis Reactivity, and Depressive-Like Behavior When Combined with Postweaning Social Isolation in Rats. PloS One 11, e0162665. https://doi.org/10.1371/journal.pone.0162665

Weaver, I.C.G., Cervoni, N., Champagne, F.A., D'Alessio, A.C., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nat. Neurosci. 7, 847–854. https://doi.org/10.1038/nn1276

Weiss, I.C., Pryce, C.R., Jongen-Rêlo, A.L., Nanz-Bahr, N.I., Feldon, J., 2004. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav. Brain Res. 152, 279–295. https://doi.org/10.1016/j.bbr.2003.10.015

Zimmerman, M., McDermut, W., Mattia, J.I., 2000. Frequency of anxiety disorders in psychiatric outpatients with major depressive disorder. Am. J. Psychiatry 157, 1337–1340. https://doi.org/10.1176/appi.ajp.157.8.1337

Chapter 4 |
Maternal separation effects on dams

Maternal separation effects on mothers – from rodents to insights in humans

Ibrahim Bölükbas [1*], Annakarina Mundorf [1*], Nadja Freund [1]

*joint first

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

Review article / submitted

Abstract

Background: The postpartum period is a sensitive time where women are especially vulnerable to develop mental disorders, like Postpartum Depression (PPD). PPD affects 10-15% of all women after parturition. Clinical aspects of the mother-infant relationship and long-term consequences of PPD on children are well studied. But the risks and etiology of PPD are still rarely analyzed. This review investigates whether the maternal separation (MS) paradigm in rodents holds potential to help understanding maternal mental health after parturition and mothers suffering from PPD. MS is a well-established stress model to investigate effects on infants, whereas potential effects on the dam are often overlooked.

Methods: PudMed was searched for studies investigating effects of daily MS within the first two weeks after parturition in rats and mice on dams and compared to findings in PPD mothers. MS was categorized as brief MS (5 min – 45min) with or without handling of pups and long MS (3h – 4h and longer). Further manipulations added to MS e.g. electric shocks or temperature alterations causing further stress on dams or offspring were excluded in this analysis.

Results: Some behavior, neurotransmitter and hormone levels seem to be altered in MS dams similar to observations in PPD patients.

Limitations: There are various protocols for conducting MS, therefore the studies are mostly not replicated or show contradictive results. Some results found in dams differ in directionality from PPD.

Conclusion: MS can provide robust information on many dimensions of behavior, neurobiology and endocrinology in the vulnerable postpartum phase and can help improve our understanding of PPD. However, some results need to be considered carefully as they differ in directionality.

Keywords: Postpartum depression, maternal mental health, Neuroanatomy, translational, Hormones, Behavior

Introduction

Postpartum depression (PPD) can develop in the 6 months following delivery and affects nearly 10-15% of women (Robertson et al., 2004; American Psychiatric Association, 2013). Main symptoms include loss of pleasure and interest, changes in eating behavior, anxiety, inability to sleep and disinterest in the baby, family, and friends (American Psychiatric Association, 2013). It is well-studied that children are also affected by maternal PPD as parameters of mother-infant relationship are impaired (Philipps and O'Hara, 1991; Letourneau et al., 2017). Disrupted regular infant engagements reduce infant's cognitive, social and emotional development with profound effects on adulthood onset (Murray and Cooper, 2013). In severe cases, PPD can result in committing suicide and even infanticide (Robertson et al., 2004). Therefore a better scientific and clinical understanding of the underlying neurobiology and exact mechanisms causing PPD is necessary. Despite its high prevalence, PPD is still under detected and undertreated (Evins et al., 2000; Gale and Harlow, 2003; Brummelte and Galea, 2016; Cox et al., 2016). As the investigation of PPD proves to be difficult in humans, animal models are useful to understand the effects on the mother's psychobiology.

As PPD is a disorder of multifactorial origin, different animal models target different aspects of the disorder. There are hormone-based models which try to mimic hormonal changes due to parturition (Aguggia et al., 2013). Others induce PPD with the Forced Swim Test (FST) similar to other depression models. Like many disease models these models only consider one side of the disorder and often neglect others. However, one other highly relevant side of PPD is impaired social interaction. Here, the maternal separation (MS) paradigm could render new insights to this social aspect of PPD. It is a stress-model in which dams are repeatedly separated from their pups over the first weeks after parturition (Zimmerberg et al., 2003; Boccia et al., 2007; Macrì et al., 2008; Kosten and Kehoe, 2010). The MS model features one key symptom of PPD mothers as in both, the separated dams and PPD patients, the main focus lies on impaired social interaction with infants during the vulnerable postpartum period (Dennis et al., 2004; Jones and Coast, 2013; Li and Chou, 2016). In MS experiments, research has mainly focused on consequences for pups neglecting possible effects on mothers (Tsuda et al., 2014). Repeated separation from their pups induces significant alterations in dam's behavior such as inducing depressive- or anxiety-like behavior (Boccia et al., 2007; Sung et al., 2010) similar to symtpoms of PPD patients. Though, it has been shown that various alterations in dams were detected (Daoura, 2010), the investigation of consequences of MS on the maternal side just recently got attention (Alves et al., 2019; Orso et al., 2019). This review proposes that the MS procedure of rodents holds potential to investigate PPD. Therefore, behavioural, hormonal and

neuroanatomical parameters will be compared. However, it is highlighted that more studies are needed to shed light on the effects of early mother-infant interaction in dams and to fully understand the phenomenon of PPD in humans (Kalinichev et al., 2000; Eklund et al., 2009; Fodor and Zelena, 2014; Pawluski et al., 2017).

There are various protocols for conducting MS (Lehmann and Feldon, 2000). In the following, daily MS within the first two weeks after parturition in rats and mice is summarized as brief MS (5 min – 45min, BMS) with or without handling of pups and long MS (3h – 4h and longer, LMS). Further manipulations added to MS e.g. electric shocks or temperature alterations causing further stress on dams or offspring were excluded in this analysis.

We hypothesize that disruptions of normal mother-pup interactions provoked by daily MS provide useful insights for PPD research. Therefore, findings that were discovered in dams after MS followed by discussions and comparisons of animal results with studies on PPD patients are presented. In general, we aim to provide an overview of the overlooked, but powerful, analysis of dams experiencing MS and how these results can help understanding maternal health after parturition.

Maternal Care

Maternal care is a highly preserved set of behavioral capacities (Leckman and Herman, 2002; Angoa-Pérez et al., 2014; Dulac et al., 2014) and alterations in maternal care might be very useful as involved pathways are associated with certain psychopathologies (Leckman and Herman, 2002). Several hypotheses regarding different styles of maternal care including its significance on adaptation have already given substantial insights into human development (Beery and Francis, 2011). Generally, neonatal stimuli are necessary to induce the expression of maternal care (Korner, 1973; Orpen and Fleming, 1987). There are well-established, objective and quantifiable parameters to assess the quality of maternal care. One crucial parameter is (I) the licking and grooming of the pups (see, e.g. (Mayer et al., 1990; Champagne et al., 2003; Weaver et al., 2004)). Further parameters to evaluate maternal care include (II) the nursing posture in either an active arched-back posture or passive posture (Champagne et al., 2003; Brunelli et al., 2015), (III) time spent with the pups, (IV) delay to collect pups as well as (V) nest-building behavior. Besides parameters assessing the time dams spent with pups, there are also self-maintenance parameters such as self-grooming and rearing that are regarded as non-pup-related behaviors to only serve the dam's needs.

When comparing maternal care after MS, most studies found an increase in maternal care with, e.g., increased licking and grooming after MS in mice and rats compared to non-separated controls (s. suppl.table 1). An increase in maternal care seems to be induced mainly

by the LMS paradigm (Pryce et al., 2001; Zimmerberg et al., 2003). However, BMS also increased maternal care upon reunion (Macrì et al., 2008; Wei et al., 2010). This indicates that increased maternal care could be an attempt of the dams to compensate for the missed time of maternal care during separation (Orso et al., 2018).

Most studies found an increase in maternal care after MS, some studies, however, are demonstrating the opposite. For example, in a study with Long-Evans rats the researchers found decreased licking and grooming of pups after LMS (Boccia and Pedersen, 2001; Boccia et al., 2007) and an overall longer time needed to retrieve pups after LMS in Sprague-Dawley (SD) and Wistar rats (Maniam and Morris, 2010; Aguggia et al., 2013). Interestingly, the latter two groups both only detected decreased maternal care when analyzing the pup retrieval times. Other groups did not find changes in maternal care showing that separated and non-separated had similar frequencies of arched-back nursing, licking and grooming and nest-building behavior after LMS (Romeo et al., 2003; Tan et al., 2017). Dams might, like humans, cope differently with stress exposure meaning separation could result in over caring or a helpless state of less caring.

Considerable variation in study design and evaluation protocol was observed when comparing the different studies. Not only MS paradigms varied in their settings (such as time being separated, the circadian time chosen for separation) but also time to record maternal care varied (during light or dark phases) (Boccia and Pedersen, 2001; Macrì et al., 2008). Results demonstrate that settings either in light phase or dark phase have a significant impact on the regulation of active nursing throughout the day (Macrì et al., 2008). Other groups followed MS procedures in which rat pups were cross-fostered into all male or all female litters (Kalinichev et al., 2000). With evidence showing that Long-Evans dams licked their male infants more frequently than female offspring (Moore, 1982), cross-fostering is likely to influence the outcome of separation.

Another interesting point is that frequently not all parameters of maternal care were increased or decreased equally across studies. For example, studies demonstrating increased arched-back nursing after MS found decreased nest-building (D'Amato et al., 1998). On one hand, this might indicate that dams focus on several parameters of maternal care whereas others are neglected. On the other hand, on-nest time might only provide a simple measure of maternal care and yet it is unclear how it relates to maternal care since there are other parameters that are more closely related to emotions, such as licking and grooming (Millstein and Holmes, 2007).

Moreover, evaluation of maternal care seems to strongly depend on the recording timeframe and the time points chosen after reunion with pups. A study comparing maternal care

after MS at several time points across the day (0900, 1145, 1400, 1600, 1900) found that within the same experimental settings the assessed parameters either showed increased or unaltered behavior depending on the time point (Pryce et al., 2001). For the parameter carrying of pups, for example, they found an increased carrying of LMS dams compared to controls at 1600, a decreased carrying at 1400, and unaltered carrying at 1600 and 1900 (Pryce et al., 2001) highlighting the variances of maternal care at several time points.

Even in studies with unaltered maternal care in MS dams, with only a limited number of observations, it might still be possible that MS dams do transiently increase their level of maternal care directed toward their pup (Romeo et al., 2003). Again, differentiating between recording time being described as "immediately after reunion" or "throughout the day" (Macrì et al., 2008) is crucial to interpreting data adequately. Here, some authors suggest that a daily analysis instead of one overall score could provide more detailed information on how the pattern of maternal care is changing after MS (Orso et al., 2018). Lastly, when comparing different studies strain-specific differences of the experimental animals need to be taken into consideration. In a study with different mice strains, an overall increased maternal care after LMS was found even though some strains, like BALB/c show general lower level of maternal care and less nest building qualities, compared to C57BL/6J (Millstein and Holmes, 2007). Surprisingly, the strains showing a lower baseline maternal care presented a significant relative change in maternal care after MS (Millstein and Holmes, 2007). This strain difference in mice might indicate that strains such as C57BL/6J mice could be more resilient to MS whereas other species (such as BALB/C mice) are more stressor-reactive and thus provide a more robust model to analyze psychopathologies of dams (Wei et al., 2010). Nevertheless, despite all variances in parameters, experimental settings, and recordings of MS, most of the studies demonstrate that MS increases maternal care upon reunion (suppl. table 1.1).

PPD in humans does not reflect the results obtained in rodents since the majority of studies demonstrated that PPD patients show more difficulties in the parenting role than nondepressed mothers indicating that many mothers struggle to cope with the responsibility of parenting (Weissman et al., 1972, 1974; Milgrom et al., 2011; Letourneau et al., 2017) and adapting to the social role of the mother (Barr, 2008). Extensive reviews on parenting behavior of PPD patients found positive as well as disengaged and negative reactions towards the child (Lovejoy and Graczyk, 2000; Weinberg et al., 2008; Field, 2010). PPD patients with positive parenting behavior demonstrated pleasant and enthusiastic maternal care; negative parenting behavior included hostile or coercive behavior (such as threatening gestures, negative facial expression) whereas in disengaged parenting behavior PPD mothers are neutral and uninvolved

towards their child (e.g., ignoring, avoiding gaze aversion) (Chabrol et al., 1996; Lovejoy and Graczyk, 2000; Paulson et al., 2006; Gruhn et al., 2016). Additionally, Field (2010) reviewed poor caregiving practices including parameters such as breastfeeding, thoughts of harming infants and punishment (Field, 2010). Included studies generally indicated that depressed mothers were more likely to use harsh penalties, more frequently thought about hurting infants and reduced odds of continuing breastfeeding (Field, 2010). This trend is also shown by other reviews indicating that for measures of detached and even harmful behavior, PPD mothers exhibited significantly higher levels of expression than nondepressed mothers (Chung et al., 2004; Knox et al., 2015). On means of positive response, depressed mothers showed considerably lower levels of maternal care than nondepressed mothers (Atkinson et al., 2000; Watkins et al., 2011; O'Hara and McCabe, 2013). However, one study also detected no changes in maternal care in PPD patients (Kim et al., 2014).

Whereas PPD mothers show contrasting maternal care compared to MS dams the disruption of maternal care is a clear similarity in both humans and rodents. This might enable a deeper insight into the physical and psychological consequences of the absence of mothers (in PPD patients) and dams (during MS experiments) influencing the behavior and neurobiology. And though the results on maternal care show contrasting effects, MS settings reflect these absences of PPD very well given that one of the main symptoms of mothers suffering from PPD is the disruption in the social dimensions (Li and Chou, 2016).

Anxiety

Anxiety is often increased in the offspring after MS (McIntosh et al., 1999; Vilela et al., 2017). Therefore, some studies evaluated anxiety in dams after MS as well. Interestingly, these studies mostly demonstrate significant but mixed results on anxiety measures. As stress and anxiety are highly correlated (Sarason, 1984; Mahmoud et al., 2012) it is no surprise that dams experiencing MS show altered anxiety as the chronic absence of pups create a stress reaction that affects behavior (Stanton et al., 1988; Fuentes et al., 2016). However, studies testing anxiety in dams show contrasting results with both more and less anxiety caused by MS when measured by the elevated plus maze (EPM) or the open field test (OFT) (see: TABLE 1.2.). Furthermore, one study could not detect clear changes in anxiety levels (Eklund et al., 2009).

In rodents, anxiety is often tested based on their avoidance behavior towards open spaces, favoring closed spaces (Pellow & File, 1986). In the EPM, animals can freely explore two closed and two open arms (Pellow et al., 1985; Mechan et al., 2002; Leo and Pamplona, 2014; Monteiro et al., 2015). Consequently, the time spent on the open arms is a measure of

anxiety with less time spent in the open indicating more anxious behavior (King, 1998). After LMS, dams show more anxiety in the EPM indicated either by less time spent in the open arms (Bousalham et al., 2013) or less open arm entries (Aguggia et al., 2013). When comparing LMS and BMS in SD rats, again LMS resulted in less time spent in the open arms (Maniam and Morris, 2010). However, two studies found an increase of time spent on open arms after separation (Kalinichev et al., 2000; Boccia and Pedersen, 2001). Here, it is interesting that both studies showing decreased anxiety tested Long Evans rats indicating that anxiety reactions after MS might be strain specific.

A very similar test is the OFT where time and entries in the center serve as measurement for anxiety (Carola et al., 2002). In line with results in the EPM, Wistar dams show less time and entries into the center after LMS and therewith increased anxiety (Bousalham et al., 2013). In a slight variation of the task where a hiding tube is provided in one corner and animals were habituated to the apparatus, no differences between dams after LMS, BMS and controls could be found (Eklund et al., 2009). This could indicate that rodent's habituation and learning in new environments (such as testing apparatus) can play a major role when interpreting measurements on anxiety limiting validity on long-term anxiety predictions (Leussis and Bolivar, 2006). Taken together, MS resulted in increased anxiety in several rat strains (suppl. Table 1.2) though strain-specific differences were noticeable. The same result can be found in PPD patients showing increased anxiety compared to healthy controls. Anxiety disorders or anxiety symptoms are often comorbid in human mothers suffering from PPD (Reck et al., 2008; Falah-Hassani et al., 2016). Rates of 57 to 100% for this comorbidity have been reported (Matthey et al., 2003). Reports highlight that anxiety with panic attacks are common in PPD (Matthey et al., 2003; Ross and McLean, 2006). Therefore, further anxiety measurements in rodents and interactions are crucial and behavioral tests after MS can enlighten our knowledge about anxiety in PPD patients clarifying about potential cross-links between depression and anxiety.

Forced Swim Test and depression

Depressive symptoms have extensively been studied in animal models providing unique molecular and translational aspects for deeper understanding of etiology and pathogenesis (Chang et al., 2011; Krishnan and Nestler, 2011). As mentioned earlier, several rodent models aim to mimic human PPD, but vary in methodology (Li and Chou, 2016) and aims.

In animal models, the Forced Swim Test (FST) is commonly used to study depressive-like behavior (Can et al., 2011; Rincón-Cortés and Sullivan, 2014; Yankelevitch-Yahav et al., 2015). As a measure of behavior despair, the rodents are tested in a tank filled with water, with fewer escape attempts and more time of immobility inducing learned helplessness and

measuring behavior despair (Bogdanova et al., 2013). Albeit not many studies have explicitly focused on studying depressions, most studies in dams found increased depressive-like behavior with only one study that found no differences on depression (suppl. table 1.2). In Wistar dams, LMS of 3 hours induces a highly significant increase of depression-like behavior with increased time of immobility (Von Poser Toigo et al., 2012; Bousalham et al., 2013) compared to non-separated controls. Furthermore, compared to BMS, it has been demonstrated that LMS particularly increases immobility in FSTs and leads to less sucrose consumption highlighting depressive-like behavior in (Boccia and Pedersen, 2001; Maniam and Morris, 2010). On the second day of FST testing LMS dams clearly showed fewer escape attempts showing learned helplessness (Boccia et al., 2007). The only study not to verify depressive-like behavior in dams after LMS was conducted with Wistar rats and separation lasted for 4.5h (Aguggia et al., 2013). This finding is in contrast with previously mentioned studies that found significant depressive-like behavior in Wistar rats after 3 hours of daily separation. Longer separation might have caused the difference in behavioral outcome (Aguggia et al., 2013). However, there is a current controversy in the field as to whether the FST is a measure of depressive-like behavior or due to passive coping responses via learning (Molendijk and de Kloet, 2015). Learned helplessness can also be induced and measured in humans and is known to induce 5 of the 9 symptoms listed by the Diagnostic and Statistical Manual of the American Psychiatric Association to diagnose depression (Maier and Seligman, 2016). As PPD is a specific form of depression, mainly diagnosed by the time of development (after giving birth), learned helplessness plays a major role to characterize the disorder. However, as the interrupted mother-infant relation is the focus of investigation of most human studies, no studies specifically focusing on helplessness or despair could be found. Therefore, testing depressive-like behavior is a crucial part when characterizing an animal model of PPD.

Changes in reward circuitry

Reward circuitry on the neuronal bases is highly relevant to psychiatric disorders in clinical practice (Chau et al., 2004; Pizzagalli et al., 2008). Data from both, human and rodent models helped to understand reward deficits in depression (Russo and Nestler, 2013). For example, studies demonstrate that alterations in reward circuitry in the central nervous system underlie drug addiction (Koob and Le Moal, 1997, 2005). In particular, changes in cocaine self-administration were found in several studies in dams after experiencing MS (Delavari et al., 2016). The length of separation appeared to not affect the dams' behavior (Moffett et al., 2006). As mentioned in measuring anhedonia in depression, non-drug measurements in changes of reward can involve sucrose consumption in different solution (10%, 5%, and 2.5% sucrose) or

as a two-bottle choice test where one bottle contains pure water and a second bottle a 10% sucrose solution to analyze altered reward behavior (Michaels and Holtzman, 2006; Michaels and Holtzmann, 2007). As sucrose consumption is significantly altered in MS separated dams, one might defer that separation stress affects brain mechanisms involved in the reward circuitry (Papp et al., 1991; Monleon et al., 1995; Kalinichev et al., 2000; Michaels and Holtzman, 2006; Michaels and Holtzmann, 2007). However, results in sucrose consumption show mixed results in MS dams (suppl. table 1.2).

Similar to depressive symptoms, PPD causes stress on the maternal side and also changes in the reward circuit leading to various comorbid psychopathologies (Nielsen et al., 2000; Josefsson et al., 2007). Some analyses revealed rapid habituation of response related to rewards in the ventral striatal region, differences in dopamine receptors such as D2/3 receptor binding potential as well as a study of dopamine system responsivity to rewarding stimuli and reward attachment networks (Moses-Kolko, 2010; Moses-Kolko et al., 2011, 2012). As in many other psychopathologies, the clarification of neurobiological changes in reward circuitry might be a future target in order to prevent comorbidities such as drug abuse in PPD women. Both MS in dams and PPD mothers show altered reward circuitry function with differences on neuronal and behavioral levels.

Memory impairments

Investigations have shown that short- and long-term stress greatly impacts cognitive and memory functions (Deffenbacher et al., 2004). In rodents, memory abilities are frequently tested using a one-trial step down inhibitory avoidance test either one or 24 hours after MS to detect short-term or long-term memory, respectively (Netto and Izquierdo, 1985; De Oliveira Alvares et al., 2005; Nasehi et al., 2012). The rodents explore the platform for a few seconds and then step down onto a grid receiving mild foot shock. During trials, the time the rodents spend on the platform before stepping down is measured (Bekinschtein et al., 2008). Impairments in both, short-term and long-term memories were reported after MS (Aguggia et al., 2013). Comparable results were obtained by Sung and colleagues (2010) who demonstrated that separating dams from their pups in LMS protocols resulted in memory impairments with lower time to step down compared to non-separated control (Sung et al., 2010).

Even though it is known that major depressive disorder (MDD) causes cognitive impairments in patients (Christensen et al., 1997; Austin et al., 2001; Lee et al., 2012) not much data is gathered on memory impairments in PPD. De Almeida and colleagues (2012) analyzed 395 individuals (222 women and 173 men) using the Edinburg Postnatal Depression Scale combined with a word span test to measure memory ability in affected PPD patients (Wadhwa

et al., 2001; Almeida and Sweeney, 2012). As shown in animal models of MS, PPD patients revealed both, impaired working and short-term memories (Almeida and Sweeney, 2012). In addition, other groups reported disrupted emotional memory in pregnant women at risk for PPD (Williams et al., 2015). Far from emotional dysregulations, PPD mothers and separated dams show impaired cognitive functions such as memory deficits and illustrate the extensive complexity of emotions and its influence on memory.

Hormones and neurotransmitter imbalances

Especially in the postpartum period, hormonal and neurotransmitter alterations play an important role. Reproductive hormones such as estrogen, oxytocin, and prolactin do not only adapt to prenatal conditions after parturition but are altered in depressive disorders as well (Soares and Zitek, 2008). Moreover, Hypothalamic-pituitary-adrenocortical (HPA) axis crosslinks with sex hormones in maternal psychiatric disorders are evident (McCormick and Mathews, 2007). Neurochemically, the released neurotransmitters of the mesolimbic system, and endogenous polypeptides in PPD patients differentiate from healthy controls highlighting the complex facets that are involved in PPD's multifactorial etiology (Bloch, 2003). Many hormonal dysregulations that appear in rodents after MS and PPD mothers might explain why a dam or PPD patient is at increased risk of impairments in social behavior towards the pup which is mediated through excreted endogenous substances (suppl table 2).

Corticosterone/Cortisol

When measuring corticosterone (CORT) concentration in the plasma, the HPA response to a stressful procedure (such as MS) is a standard measurement of stress reaction (Levine, 2000, 2005; Möstl and Palme, 2002; Sheriff et al., 2011). Most MS experiments resulted in an increase of plasma CORT after BMS (D'Amato et al., 1992) and LMS (Zarrow et al., 1972; Nagy et al., 1983; Maniam and Morris, 2010) (suppl table 2). Furthermore, relative differences within separation durations were detected. Results demonstrate that LMS induced higher CORT concentrations then BMS in SD dams (Maniam and Morris, 2010) implying that LMS can even cause greater stress in these dams. Contrary to this finding, others found higher CORT concentrations in BMS compared to LMS in Wistar dams (Eklund et al., 2009). One possible explanation for these results might be explained by differences in habituation to the stressful procedure of separation showing that SD rats habituate faster to shorter separation whereas Wistar rats show slower habituation and thus show greater stress response (Eklund et al., 2009). Moreover, adrenal gland and body weights decreased despite the increases of cortisol in BMS dams (Eklund et al., 2009). This might suggest that in particular prolonged separation causes

stress in dams' physiological parameters as adrenal hypertrophy (and not hypotrophy) is seen in chronically stressed animals (Ulrich-Lai et al., 2006).

Few studies found contrasting result. Some groups even did not find significant differences in CORT levels (Wei et al., 2010). Interestingly, this study was the only one conducted with BALB/c mice in a BMS paradigm. Firstly, it must be highlighted that CORT measurements were assessed 3 hours after reunion (instead of instantaneous measurements) which might indicate that assessment time plays a crucial role with physiological adaptation and downregulation of stress hormones upon reunion. Secondly, this is the only study involving mice which might indicate that CORT level measurements in mice might be difficult. Moreover, others have shown that BMS even decreased the basal serum CORT concentrations (Leuner et al., 2007). Importantly, this study only compared single separation of pups compared to controls that were able to nurse pups. Compared to the chronic separation paradigm that mostly increases corticosterone levels, one might argue that basal levels of circulating CORT are high after giving birth and this elevation requires the presence of nursing pups to maintain high levels (Korányi, 1977; O'Malley et al., 2008). Single separation of pups from the dam results in less pup-presence and consequently decreases basal CORT concentrations (Leuner et al., 2007). But the differences between the basal and stress-induced cortisol levels given in the literature need to be considered carefully as again, interindividual differences to stress stimuli are known.

Endocrinological changes occur during pregnancy and exert a strong influence on mother's mood and behavior (Altshuler et al., 1998; Janssen et al., 2016). Interestingly, reports show that mothers with higher cortisol levels demonstrated increased preference and affection towards the infants' emotions (Orpen and Fleming, 1987; Fleming et al., 1997; Olza-Fernández et al., 2014). In a systematic review on PPD and changes in cortisol levels, 47 studies were analyzed with 24 studies reporting significant associations between cortisol level and depressive symptoms, whereas 23 studies did not find significant associations (Seth et al., 2016). Measurements of cortisol were taken from blood samples, saliva, or urine probes. Therefore, quality of cortisol measurements and outcome measures strongly varied between studies (Schiller et al., 2015; Seth et al., 2016). Moreover, HPA axis dysfunction is implicated in the pathogenesis of PPD and HPA axis is common in neuroendocrinal alterations found in depression (Heim et al., 2008; Pariante and Lightman, 2008). New approaches involving biomarkers in early-detection of PPD where cortisol, as a stress-hormone, plays a crucial role and this concept bears merit (Serati et al., 2016). For most dams, MS represents a stressful event and thus, increases stress hormones as an adaptation (McEwen, 1998). Clearly, this has also

been observed in PPD patients. However, measuring cortisol levels in PPD patients' needs further establishment before serving as diagnostic tool to reliably predict and diagnose PPD based on cortisol levels.

Prolactin

Prolactin's main role in galactopoiesis and lactogenesis are elementary after parturition (Kanyicska et al., 2000; Fitzgerald and Dinan, 2008; Cabrera-Reyes et al., 2017). However, it is well known that prolactin's recently discovered functions in neurodevelopment, learning and memory which shed light on the diversity of regulatory cognitive functions beyond the classical postnatal functions (Cabrera-Reyes et al., 2017; Camacho-Arroyo et al., 2018). Regarding prolactin alterations in rodents during MS, a decrease of plasma prolactin was observed in LMS experiments (Zarrow et al., 1972; Nagy et al., 1983). In humans, prolactin is decreased in depressed mothers who were less likely to be breastfeeding (T. Abou-Saleh et al., 1998; Groer and Morgan, 2007; Schiller et al., 2015). Given prolactin's role in cognitive functions including maternal emotionality, imbalances in peripheral prolactin might lead to psychopathologies including PPD (Dondi et al., 1991; Larsen and Grattan, 2012; Torner, 2016). Both rodent models and humans show decreased prolactin levels after stress caused by either MS or as it occurs during PPD. Given prolactin's highly interesting role in social and neuronal development, further studies in the regulation of maternal psychopathology remain to be seen.

Estrogen

Although estrogen plays a crucial role in fertility, psychopathologies such as mood and anxiety disorders involving estrogen dysregulations have been reported (McEwen, 1998; Heldring et al., 2007; Sakaki and Mather, 2012; Wheelan et al., 2018). Interactions with other hormones show that estrogen receptor (ER) activation acts to induce oxytocin receptor expression. With higher ER expression, higher oxytocin receptor expression in brain regions was detected potentially changing maternal behavior (Pedersen et al., 1994). Manipulations of estrogen in animals especially affect dendritic composition and density on pyramidal neurons of the medial prefrontal cortex (PFC) (Jacobs and D'Esposito, 2011; Shanmugan and Epperson, 2014). This might indirectly influence parenting, working memory, cognitive flexibility and mood regulation in both humans and animals (Hernández-González et al., 2005; Numan and Stolzenberg, 2009; Leuner et al., 2010). Furthermore, studies of ER β showed its crucial role in the modulation of stress reactions including anxiety-related behaviors (Tsuda et al., 2014). Given its crucial role in altering mood, it is believed that the estrous cycle of dams needs to be considered in experimental settings to elucidate disruptions related to estrogen (Frye et al.,

2000; D'Souza and Sadananda, 2017) showing alterations in depression- and anxiety-like behavior. However, the reproductive stages are often characterized by cessation of ovarian cycle (Leuner and Shors, 2006). This consideration can create more comparable data even though some reports show that the estrous cycle neither influences social behavior nor locomotion in female rats (Nofrey et al., 2008; Faraji et al., 2018). Alterations in estrogen should be taken into consideration in animal studies given its interactions on psychological and physiological homeostasis and thus, should be studied more extensively to clarify its role.

Estrogen and progesterone studies in humans have shown associations between steroid hormones and mood alterations in postpartum women (Heidrich et al., 1994; Buckwalter et al., 1999). As reviewed by Schiller and colleagues (2015), both estrogen and progesterone as reproductive hormones modulate Oxytocin mRNA expression in human brain regions associated with maternal behavior and lactation (Schiller et al., 2015). So, alterations in estrogen are likely to change the relationship between mother and infant, similar to disruptions of this bond occurring in PPD (Murray and Cooper, 2013). Other approaches involve analyzing prediction of PPD with blood DNA methylation that reflect estrogen-mediated epigenetic changes (Guintivano et al., 2014). In addition to diagnostic approaches, therapeutic estrogen supplementation has been applied in clinical trials to improve symptoms of PPD and might be another possible therapeutic strategy to tackle the disorder (Ahokas et al., 2001).

Serotonin-System

The "serotonin hypothesis" of clinical depression is almost 50 years old, and still, the neurobiology of serotonin plays a crucial role in the pathophysiology of depression (Cowen, 2015) and PPD (Anderson and Maes, 2013). Animal studies have shown anxiolytic-like effects in dams after LMS with 8–OH–DPAT (serotonergic agonist) implying a role in anxiety-development in MS experiments (Picazo et al., 2000). The depression-like symptoms in rats after LMS found by Sung and colleagues (2010), showed that these changes might be mediated through decreased expressions of serotonin (5-hydroxytryptamine) and tryptophan hydroxylase in the dorsal raphe nucleus similar to other clinical depression findings (Sung et al., 2010).

In PPD, Selective Serotonin Reuptake Inhibitors (SSRI) are successfully prescribed emphasizing the crucial role in the development of PPD (Stewart, 2013). Furthermore, positron emission tomography (PET) studies underline the role of the serotonin transporter changes (Moses-Kolko et al., 2008) including depressive responses to sex-steroid hormone manipulation as these hormones fluctuate during pregnancy and postpartum causing maternal mental disorders (Frokjaer et al., 2015). Further approaches involve measurements of peripheral blood's platelet serotonin levels obtained with immunocytochemical tests for depression

scoring (Maurer-Spurej et al., 2007). Therefore, the serotonin-system might show its validity in both animal models and humans affecting the stress responses and development of mood disorders.

Endocannabinoid and Opioid system

Opioids and cannabinoids are both involved in antinociception and drug addiction showing biochemical and pharmacological interactions (Manzanares et al., 1999). The 'endocannabinoid system' (ECS) is present in the central nervous system and (besides pain management) takes part in regulating addiction, stress coping and fertility (Di Marzo, 2008; Mouslech and Valla, 2009). Amongst the cannabinoid receptors (CB) e.g. the CB1 receptor is expressed in regions regulating many key functions, including cognition and mood regulation (Mackie, 2005; Cannizzaro and Diana, 2016) with abnormalities in density found in psychiatric disorders (Wong et al., 2010; Patel et al., 2017). Another relevant group of receptors are CB2 receptors which are widely distributed in peripheral tissues (Svíženská, 2008). Schechter and colleagues (2012) revealed the critical role of cannabinoids in mediating maternal care: the blockage of the endocannabinoid system such as the mouse dam's CB1 receptors resulted in less maternal care with less pup retrieval and crouching over pups (Schechter et al., 2012). Furthermore, Kalinichev and colleagues (2000) found decreased sensitivity to antinociceptive qualities of morphine and hyperalgesia, especially in LMS (Kalinichev et al., 2000). It is likely that repeated prolonged separations from the litter, or the return of the offspring after the separation, may disrupt homeostasis between the endogenous opioids and maternal neural circuit, triggering long-lasting changes in both systems (Kalinichev et al., 2000).

Considerable evidence suggests the involvement of the ECS in neuroendocrine and inflammatory pathways involved in depression and pain, which might explain an 80% comorbidity rate (Fitzgibbon et al., 2016). Therefore, it is not surprising that altered endocannabinoid signaling is not only detected in patients with chronic pain but also in psychiatric patients (Di Marzo, 2008; Huang et al., 2016). Eisenach and colleagues (2008) suggest that pain experience in mothers during parturition can be a crucial element in the development of PPD with the severity of acute pain after childbirth predicting persistent pain and PPD development (Eisenach, 2008). Yim and colleagues (2010) suggest that prenatal β-endorphin might serve as an early predictor of PPD (Yim et al., 2010). Social stimuli release opioids and regulate the rewarding system and social motivation highlighting the importance in the infant-mother attachment which is altered during PPD (Nelson, 1998).

The cannabinoid and opioid systems are both evident in human and rodents with functions that reach far beyond pain management but also in mediating maternal role.

Therefore, MS can be extremely useful to enlighten our understanding of these systems in maternal care and PPD.

Neuroanatomy

A profound understanding of the neuroanatomy of PPD could help determining whether specific regions in the brain might contribute to mood disorders and changes in social behavior. Structures involved in the limbic system are playing a significant role in emitting endorphins, the endogenous opioids of humans (Sweep, 1989). Thus, the alteration of central structures of the mesolimbic system such as the hippocampus and the amygdala's activation in maternally separated dams and mothers with PPD is no surprise. Further insights can help us provide a more differentiated picture of psychopathology of PPD resulting not only in mood changes but in a more aggressive or less detached mothers (suppl. table 3).

Hypothalamus

As addressed earlier, perinatal HPA-axis dysregulation is associated with stress and the development of PPD (Glynn et al., 2013). Corticotropin-Releasing Hormone (CRH) is a peptide released from the Hypothalamus to stimulate the synthesis of Adrenocorticotropic hormone (ATCH) in the pituitary gland (Allen and Sharma, 2019). Maniam and colleagues (2010) analyzed hypothalamic excretion of polypeptides that mediate HPA-axis with higher CRH mRNA in MS dams (Maniam and Morris, 2010). This up regulation was higher in LMS compared to BMS dams, emphasizing that prolonged chronic absence of pups markedly affected not only behavioral but also neuroendocrinological parameters (Maniam & Morris, 2010). Furthermore, the hypothalamus secretes neuropeptide Y (NPY) that was reduced in this LMS study is supporting the possible role of hypothalamic NPY in creating anxiety-like behavior and depressive-like behavior. Therefore, changes in these hypothalamic systems, as shown in the dams after LMS in rats, are likely to explain differences in both anxiety- and depressive-like behavioral dimensions after stress (Heilig, 2004).

Both, CRH and NPY were also analyzed in PPD patients showing clear alterations. Analyses of secreted CRH in PPD patients showed increased hypothalamic CRH (Magiakou et al., 1996). Other groups measured increased placental CRH concentrations as placenta also produces significant CRH amounts (Schulte et al., 1990). In particular, during the third trimester placental CRH shows strong correlations with the evolvement of PPD (Bloch, 2003; Yim et al., 2010). Also, in humans, the NPY system of the hypothalamus is known to modulate stress, anxiety, and depression (Redrobe et al., 2002). Xie and colleagues (2018) recently observed decreased NPY levels in human plasma (Xie et al., 2018) similar to the finding of decreased

NPY in MS rat dams. But especially for brain's NPY secretion, its role in PPD needs further investigation (Redrobe et al., 2002) due to the variety of behavioral modulations. The hormones CRH and NPY secreted by the hypothalamus of dams and PPD patients provide insights in stress modulation response on central nervous level. Here, animal models can be especially helpful to study neuronal alterations on a molecular level.

Hippocampus

Neuroplastic changes during the postpartum period in the maternal brain are influenced by the interaction with their pups (Bridges and Scanlan, 2005; Galea et al., 2006; Kinsley and Lambert, 2008; Olza-Fernández et al., 2014; Opendak et al., 2016). Several experiments have found hippocampus alterations in pups and dams showing both increased and decreased neuronal cell growth. Leuner and colleagues (2007) have shown that one-time BMS in SD dams prevented a decrease in cell growth rate in the hippocampus (Leuner et al., 2007). It must be highlighted that in this setting pups were only separated one time instead of repeatedly. Another study found alterations in the hippocampus after dams experiencing repeated LMS with a decrease of cell proliferation and an increase of apoptosis (Sung et al., 2010). However, these results after BMS and LMS seem to be contradicting. One possible explanation could be that short and single MS settings might not be enough for causing stress levels that lead to an adaptive and significant CORT rise which then decreases cell proliferation. Thus, increased cell proliferation of hippocampal cells is plausible in BMS, whereas more extended MS impairs and diminishes hippocampus neurons (Leuner et al., 2007). Besides cell proliferation studies, analyses of stress reactions in the hippocampus found lower glucocorticoid-receptor (GR) mRNA expression in dams, especially in LMS compared to BMS (Maniam and Morris, 2010). Given that GR mediates the direct effects of glucocorticoid release in response to stress and regulates the HPA system through negative feedback, decreased hippocampal GR mRNA is consistent with the elevated plasma CORT levels as a stress response (Maniam and Morris, 2010). New approaches involving biomarkers to detect women at risk of developing depressive symptoms during pregnancy take advantage of increased peripheral GR mRNA expression (Katz et al., 2012; Engineer et al., 2013). Further MS experiments on dams' hippocampus revealed lower Natrium, Kalium-ATPase activity and NO-activity emphasizing the neurochemical alterations in the hippocampus that are related to depression (Von Poser Toigo et al., 2012).

Cross-links of alterations in stress hormones and neuronal plasticity are well-studied in animals and humans with steroids as a key player of regulating corticolimbic system (Berman et al., 1997). Differences of neuroactive steroids were studied extensively and demonstrated

significant alterations especially in the regions of the anterior cingulate cortex, amygdala, hippocampi, and dorsolateral PFC (Deligiannidis et al., 2013). However, there are relatively few studies examining the neurobiology of PPD patients though it is recognized that the postpartum period in general is accompanied by neuroplastic changes in the hippocampus and PFC (Brummelte and Galea, 2016).

Amygdala

The role of the amygdala in humans involves the detection of threat and modulation of fear (Öhman, 2005) as well as social judgments (Adolphs et al., 1998). In rodents, the amygdala appears to modulate fear and anxiety (as discussed in *Anxiety*), and plays a role in memory and attention, in sexual and sex-related behavior of rats (Rasia-Filho et al., 2000), as well as in neuronal plasticity during motherhood (Rasia-Filho et al., 2004). In an animal study, LMS yielded high c-Fos expression in the central nucleus of the amygdala providing further evidence for neurobehavioral effects of MS in dams (Aguggia et al., 2013).

Reviews resuming imaging studies in humans show exciting insights into the amygdala as part of the "maternal caregiving network" (Pawluski et al., 2017). As mentioned earlier, studies show that neuroactive steroids affect the amygdala in PPD (Chase et al., 2014). Engagement of functional magnetic resonance imaging (fMRI) studies demonstrate disrupted posterior cingulate–amygdala connectivity (Deligiannidis et al., 2013; Kim et al., 2016). Further imaging techniques could provide more profound insights into the neurobiological changes in PPD. PET-studies in patients with PPD analyzed neurobiological changes showed differences in the amygdala and the hippocampus as part of the mesotemporal cortex with lower serotonin 1A receptor numbers (Moses-Kolko et al., 2008).

Discussion

PPD is a highly prevalent mental health disorder. For biological psychiatry, animal models have proven to be very successful (Krishnan & Nestler, 2011). Though different animal models for PPD are introduced and range from hormone-based approaches to classical depression models such as LH, the MS paradigm can offer the potential to understand neurobiological and hormonal changes of PPD that are caused by social disruption. We believe MS might present a neglected and powerful tool to increase our knowledge on PPD and maternal mental health after parturition recognizing its primary development aimed to study infants' outcomes after MS. As in all animal models for studying PPD, limitations must be considered.

As seen in the case of maternal care, the findings of an evolutionary crucial parameter (Royle, Smiseth, & Kölliker, 2012) emphasizing significance of this mediator in psychopathological development. On anxiety and depression tested in dams after experiencing MS from the pups, most studies showed increased anxiety and depression-like behavior (s. table 1.2). This is in line with findings in PPD patients compared to control mothers. Interesting results were obtained with aggression showing decreased aggression whereas humans of PPD showed increased aggression levels leading to even harmful behavior towards oneself or towards the infant. Changes in reward circuitry that can enlighten our understanding of comorbidities, such as drug addiction, after stressful life events are important to investigate. Especially newer approaches with audio analyses to understand affect associated with positive stimuli will hopefully show the biological drivers of altered reward circuitry not only for PPD but also for other human psychiatric diseases that are triggered by stressful events.

Analyzing physiological reactions and neurobiological chances offer a unique benefit to corroborate findings from behavior. CORT as an established stress hormone (Heinrichs, Baumgartner, Kirschbaum, & Ehlert, 2003) showed significant results in plasma and serum confirming MS poses stress to dams with respective adaptation. The analysis of hormonal changes such as CORT, OXT, Prolactin and Estrogen after MS or PPD are important to investigate further but have generally proven to be difficult in regard of measurement and assessment (blood, cerebrospinal fluid, salvia), so far.

Neurochemical analysis of neurotransmitters including serotonin, noradrenaline, glutamate, GABA, and dopamine are crucial as these neurotransmitter imbalances are major drivers of developing depressive symptoms and are targets of most antidepressants for treating MDD and PPD patients. Neuroanatomical alterations as analyzed in MS dams are very useful as several similarities have proven to be successful in translational psychiatry (Baumann & Rothman, 1998) with most crucial neuronal structures that serve comparable functions in rodents and humans. Especially the neurogenesis process after MS and parturition itself is extremely interesting showing dynamics of neuronal adaptation after stress. Even though MS shields light on some highly interesting aspects of the biological and behavioral consequences similar to these found in PPD, they have to be considered carefully. In general, most of the results obtained from MS experiments so far, need further validation in more explicit test settings and in a more careful consideration of testing parameters.

Therefore, methodological issues and data collection should be aligned across studies and examine more consistently. For example, regarding maternal care, the selection of certain analyzed postnatal days is not explained. This also refers to time of recording frame varying

from 15 minutes to 6 hours of total recording for each day. Therewith, we suggest that more detailed protocols with longer recording times and frequencies, can give useful results on behavior. This should also include cross sections for each day combined with a longitudinal analysis to examine variances in maternal behavior. Studies should further include other anxiety behavioral measurements as a battery. Further anxiety measurements such as marble burying where rodents do not show habituation (Njung'e & Handley, 1991) could complement anxiety findings. Furthermore, most studies are measuring anxiety by the end of the period whereas the timeframe during separations were often not considered. Therefore, first approaches might consider measuring anxiety during separation experiments which might give further evidence of anxious state that is created by MS. This could easily be substantiated with Ultrasonic Vocalization (USV) detection (Brudzynski, 2015) as USVs for analyzing affective state are already common in pharmacological studies with animals (Burgdorf, Wood, Kroes, Moskal, & Panksepp, 2007; Iijima & Chaki, 2005). Etiological aspects of why depression leads to memory impairments are still unelucidated (Castaneda, Tuulio-Henriksson, Marttunen, Suvisaari, & Lönnqvist, 2008). Studies in animals show clear results that are comparable to humans – a great step into showing significant results on memory impairments on both rodent and human side. But on both sides, more studies need to be conducted in order to characterize interactions of affective and cognitive disorders.

To summarize, MS is a useful stress models that can render useful insights into disrupted maternal health after parturition with a strong focus on disrupted maternal-infant bond. This model clearly causes stress on dams' side, resulting in altered behaviors that mimic psychiatric disorders such as depressive-like behavior and anxiety-like behaviors. It must be considered that there is no single MS model for rodents but several with different species, generating different results and with BMS and LMS resulting in contrasting behavioral outcomes.

Conclusion

MS can provide robust information on many dimensions of behavior, neurobiology, and endocrinology in the vulnerable postpartum phase of dams. Thus, our knowledge on psychopathologies manifesting during this vulnerable phase, such as PPD, can be improved by investigating the effects of MS on dams.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

All authors contributed equally and approved the manuscript.

Acknowledgments

We would like to thank Dr. Chan Phetsouphanh for proof-reading the manuscript. Moreover, we acknowledge support by the DFG Open Access Publication Funds of the Ruhr-Universität Bochum.

Abbreviations

ATCH: Adrenocorticotropic hormone

BMS: brief maternal separation

CB: Cannabinoid receptors

CORT: Corticosterone

CRH: Corticotropin-Releasing Hormone

ECS: Endocannabinoid system

EPM: Elevated Plus Maze

ER: Estrogen receptor

FMRI: Functional magnetic resonance imaging

FST: Forced swim test

GR: Glucocorticoid receptor

HPA: Hypothalamic-pituitary-adrenocortical

LMS: Long maternal separation

MDD: Major Depressive Disorder

MS: Maternal separation

NPY: Neuropeptide Y

OFT: Open Field Test

PET: Positron emission tomography

PFC: Prefrontal Cortex

PPD: Postpartum Depression

SD: Sprague-Dawley

SSRI: Selective Serotonin Reuptake Inhibitors

USV: Ultrasonic vocalization

References

Adolphs, R., Tranel, D., and Damasio, A. R. (1998). The human amygdala in social judgment. *Nature*. doi:10.1038/30982.

Aguggia, J. P., Suárez, M. M., and Rivarola, M. A. (2013). Early maternal separation: neurobehavioral consequences in mother rats. *Behav. Brain Res.* 248, 25–31. doi:10.1016/j.bbr.2013.03.040.

Ahokas, A., Kaukoranta, J., Wahlbeck, K., and Aito, M. (2001). Estrogen deficiency in severe postpartum depression: Successful treatment with sublingual physiologic 17β-estradiol: A preliminary study. *J. Clin. Psychiatry*. doi:10.4088/JCP.v62n0504.

Allen, M. J., and Sharma, S. (2019). "Physiology, Adrenocorticotropic Hormone (ACTH)," in *StatPearls* (Treasure Island (FL): StatPearls Publishing). Available at: http://www.ncbi.nlm.nih.gov/books/NBK500031/ [Accessed March 9, 2019].

Almeida, J. B., and Sweeney, J. B. (2012). Working and short-term memories are impaired in postpartum depression. *Tetrahedron Lett.* 39, 8703–8706. doi:10.1016/j.jad.2011.09.031.

Altshuler, L. L., Hendrick, V., and Cohen, L. S. (1998). Course of mood and anxiety disorders during pregnancy and the postpartum period. *J. Clin. Psychiatry*. doi:10.1097/00004714-199602000-00015.

Alves, R. L., Portugal, C. C., Summavielle, T., Barbosa, F., and Magalhães, A. (2019). Maternal separation effects on mother rodents' behaviour: A systematic review. *Neurosci. Biobehav. Rev.* doi:10.1016/j.neubiorev.2019.09.008.

American Psychiatric Association (2013). *Diagnostic and statistical manual of mental disorders (DSM-5®)*. American Psychiatric Pub.

Anderson, G., and Maes, M. (2013). Postpartum depression: Psychoneuroimmunological underpinnings and treatment. *Neuropsychiatr. Dis. Treat.* 9, 277–287. doi:10.2147/NDT.S25320.

Angoa-Pérez, M., Kane, M. J., Sykes, C. E., Perrine, S. A., Church, M. W., and Kuhn, D. M. (2014). Brain serotonin determines maternal behavior and offspring survival. *Genes Brain Behav.* 13, 579–591. doi:10.1111/gbb.12159.

Atkinson, L., Paglia, A., Coolbear, J., Niccols, A., Parker, K. C. H., and Guger, S. (2000). Attachment security. *Clin. Psychol. Rev.* 20, 1019–1040. doi:10.1016/S0272-7358(99)00023-9.

Austin, M. P., Mitchell, P., and Goodwin, G. M. (2001). *Cognitive deficits in depression: Possible implications for functional neuropathology*. doi:10.1192/bjp.178.3.200.

Barr, J. A. (2008). Postpartum depression, delayed maternal adaptation, and mechanical infant caring: A phenomenological hermeneutic study. *Int. J. Nurs. Stud.* doi:10.1016/j.ijnurstu.2006.10.002.

Beery, A. K., and Francis, D. D. (2011). *Adaptive significance of natural variations in maternal care in rats: A translational perspective*. doi:10.1016/j.neubiorev.2011.03.012.

Bekinschtein, P., Cammarota, M., Izquierdo, I., and Medina, J. H. (2008). Reviews: BDNF and memory formation and storage. *The Neuroscientist* 14, 147–156.

Berman, K. F., Schmidt, P. J., Rubinow, D. R., Danaceau, M. A., Van Horn, J. D., Esposito, G., et al. (1997). Modulation of cognition-specific cortical activity by gonadal steroids: a positron-emission tomography study in women. *Proc. Natl. Acad. Sci. U. S. A.* 94, 8836–41. doi:10.1073/pnas.94.16.8836.

Bloch, M. (2003). Endocrine Factors in the Etiology of Postpartum Depression. 44, 234–246. doi:10.1053/comp.2003.50014.

Boccia, M. L., and Pedersen, C. A. (2001). Brief vs. long maternal separations in infancy: Contrasting relationships with adult maternal behavior and lactation levels of aggression and anxiety. *Psychoneuroendocrinology* 26, 657–672. doi:10.1016/S0306-4530(01)00019-1.

Boccia, M. L., Razzoli, M., Prasad Vadlamudi, S., Trumbull, W., Caleffie, C., and Pedersen, C. A. (2007). Repeated long separations from pups produce depression-like behavior in rat mothers. *Psychoneuroendocrinology* 32, 65–71. doi:10.1016/j.psyneuen.2006.10.004.

Bogdanova, O. V., Kanekar, S., D'Anci, K. E., and Renshaw, P. F. (2013). Factors influencing behavior in the forced swim test. *Physiol. Behav.* 118, 227–239. doi:10.1016/j.physbeh.2013.05.012.

Bousalham, R., Benazzouz, B., Hessni, A. E., Ouichou, A., and Mesfioui, A. (2013). Maternal Separation Affects Mothers ' Affective and Reproductive Behaviors as Well as Second Offspring ' s Emotionality. *Test* 2013, 409–414. doi:http://dx.doi.org/10.4236/jbbs.2013.35042.

Bridges, R. S., and Scanlan, V. F. (2005). Maternal memory in adult, nulliparous rats: Effects of testing interval on the retention of maternal behavior. *Dev. Psychobiol.* 46, 13–18. doi:10.1002/dev.20038.

Brummelte, S., and Galea, L. A. M. (2016). Postpartum depression: Etiology, treatment and consequences for maternal care. *Horm. Behav.* 77, 153–166. doi:10.1016/j.yhbeh.2015.08.008.

Brunelli, S. A., Curley, J. P., Gudsnuk, K., Champagne, F. A., Myers, M. M., Hofer, M. A., et al. (2015). Variations in maternal behavior in rats selected for infant ultrasonic vocalization in isolation. *Horm. Behav.* 75, 78–83. doi:10.1016/j.yhbeh.2015.08.007.

Buckwalter, J. G., Stanczyk, F. Z., McCleary, C. A., Bluestein, B. W., Buckwalter, D. K., Rankin, K. P., et al. (1999). Pregnancy, the postpartum, and steroid hormones: effects on cognition and mood [published erratum appears in Psychoneuroendocrinology 1999 Jul;24(5):581]. *Psychoneuroendocrinology* 24, 69–84. doi:10.1136/annrheumdis-2012-202476.

Cabrera-Reyes, E. A., Limón-Morales, O., Rivero-Segura, N. A., Camacho-Arroyo, I., and Cerbón, M. (2017). *Prolactin function and putative expression in the brain*. doi:10.1007/s12020-017-1346-x.

Camacho-Arroyo, I., González-Arenas, A., Jiménez-Arellano, C., Morimoto, S., Galván-Rosas, A., Gómora-Arrati, P., et al. (2018). Sex hormone levels and expression of their receptors in lactating and lactating pregnant rats. *J. Steroid Biochem. Mol. Biol.* 178, 213–220. doi:10.1016/j.jsbmb.2017.12.015.

Can, A., Dao, D. T., Arad, M., Terrillion, C. E., Piantadosi, S. C., and Gould, T. D. (2011). The Mouse Forced Swim Test. *J. Vis. Exp.* doi:10.3791/3638.

Cannizzaro, C., and Diana, M. (2016). "Cannabis and the Mesolimbic System," in *Neuropathology of Drug Addictions and Substance Misuse* (Elsevier), 795–803. doi:10.1016/B978-0-12-800213-1.00074-2.

Carola, V., D'Olimpio, F., Brunamonti, E., Mangia, F., and Renzi, P. (2002). Evaluation of the elevated plus-maze and open-field tests for the assessment of anxiety-related behaviour in inbred mice. *Behav. Brain Res.* 134, 49–57. doi:10.1016/S0166-4328(01)00452-1.

Chabrol, H., Bron, N., and Camus, J. L. (1996). Mother-infant and father-infant interactions in postpartum depression. *Infant Behav. Dev.* 19, 149–152. doi:10.1016/S0163-6383(96)90053-3.

Champagne, F. A., Francis, D. D., Mar, A., and Meaney, M. J. (2003). Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* 79, 359–371. doi:10.1016/S0031-9384(03)00149-5.

Chang, C.-C., Yu, S.-C., McQuoid, D. R., Messer, D. F., Taylor, W. D., Singh, K., et al. (2011). Reduction of dorsolateral prefrontal cortex gray matter in late-life depression. *Psychiatry Res. Neuroimaging* 193, 1–6. doi:10.1016/j.pscychresns.2011.01.003.

Chase, H. W., Moses-Kolko, E. L., Zevallos, C., Wisner, K. L., and Phillips, M. L. (2014). Disrupted posterior cingulate-amygdala connectivity in postpartum depressed women as measured with resting BOLD fMRI. *Soc. Cogn. Affect. Neurosci.* 9, 1069–1075. doi:10.1093/scan/nst083.

Chau, D. T., Roth, R. M., and Green, A. I. (2004). The neural circuitry of reward and its relevance to psychiatric disorders. *Curr. Psychiatry Rep.* 6, 391–399.

Christensen, H., Griffiths, K., Mackinnon, A., and Jacomb, P. (1997). A quantitative review of cognitive deficits in depression and Alzheimer-type dementia. *J. Int. Neuropsychol. Soc. JINS*.

Chung, E. K., McCollum, K. F., Elo, I. T., Lee, H. J., and Culhane, J. F. (2004). Maternal depressive symptoms and infant health practices among low-income women. *Pediatrics* 113, e523-529.

Cowen (2015). What has serotonin to do with depression? *Electroencephalogr. Clin. Neurophysiol. Evoked Potentials* 62, 177–183. doi:10.1016/0168-5597(85)90012-7.

Cox, E. Q., Sowa, N. A., Meltzer-Brody, S. E., and Gaynes, B. N. (2016). The perinatal depression treatment cascade: Baby steps toward improving outcomes. *J. Clin. Psychiatry*. doi:10.4088/JCP.15r10174.

D'Amato, Cabib, S., Ventura, R., and Orsini, C. (1998). Long-term effects of postnatal manipulation on emotionality are prevented by maternal anxiolytic treatment in mice. *Dev. Psychobiol.* 32, 225–234. doi:10.1002/(SICI)1098-2302(199804)32:3<225::AID-DEV6>3.0.CO;2-Q.

D'Amato, F. R., Cabib, S., Puglisi-Allegra, S., Patacchioli, F. R., Cigliana, G., Maccari, S., et al. (1992). Effects of acute and repeated exposure to stress on the hypothalamo-pituitary-adrenocortical activity in mice during postnatal development. *Horm. Behav.* 26, 474–485. doi:10.1016/0018-506X(92)90015-N.

Daoura (2010). Postpartum behavioral profiles in Wistar rats following maternal separation - altered exploration and risk-assessment behavior in MS15 dams. *Front. Behav. Neurosci.* 4, 1–7. doi:10.3389/fnbeh.2010.00037.

De Oliveira Alvares, L., De Oliveira, L. F., Camboim, C., Diehl, F., Pasqualini Genro, B., Bispo Lanziotti, V., et al. (2005). Amnestic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiol. Learn. Mem.* doi:10.1016/j.nlm.2004.10.002.

Deffenbacher, K. A., Bornstein, B. H., Penrod, S. D., and McGorty, E. K. (2004). A Meta-Analytic Review of the Effects of High Stress on Eyewitness Memory. *Law Hum. Behav.* 28, 687–706. doi:10.1007/s10979-004-0565-x.

Delavari, F., Sheibani, V., Esmaeili-Mahani, S., and Nakhaee, N. (2016). Maternal Separation and the Risk of Drug Abuse in Later Life. *Addict Health* 8, 107–14.

Deligiannidis, K. M., Sikoglu, E. M., Shaffer, S. A., Frederick, B., Svenson, A. E., Kopoyan, A., et al. (2013). GABAergic neuroactive steroids and resting-state functional connectivity in postpartum depression: A preliminary study. *J. Psychiatr. Res.* 47, 816–828. doi:10.1016/j.jpsychires.2013.02.010.

Dennis, C. L. E., Janssen, P. A., and Singer, J. (2004). Identifying women at-risk for postpartum depression in the immediate postpartum period. *Acta Psychiatr. Scand.* doi:10.1111/j.1600-0447.2004.00337.x.

Di Marzo, V. (2008). Targeting the endocannabinoid system: To enhance or reduce? *Nat. Rev. Drug Discov.* 7, 438–455. doi:10.1038/nrd2553.

Dondi, D., Maggi, R., Panerai, A. E., Piva, F., and Limonta, P. (1991). Hypothalamic Opiatergic Tone During Pregnancy, Parturition and Lactation in the Rat. *Neuroendocrinology* 53, 460–466. doi:10.1159/000125758.

D'Souza, D., and Sadananda, M. (2017). Estrous Cycle Phase-Dependent Changes in Anxiety- and Depression-Like Profiles in the Late Adolescent Wistar-Kyoto Rat. *Ann. Neurosci.* 24, 136–145. doi:10.1159/000477151.

Dulac, C., O'Connell, L. A., and Wu, Z. (2014). Neural control of maternal and paternal behaviors. *Science* 345, 765–770. doi:10.1126/science.1253291.

Eisenach, J. C. (2008). Severity of Acute Pain After Childbirth, but not Type of Delivery predicts persistent pain and postpartum depression. 53, 131–149. doi:10.1016/j.pain.2008.07.011.Severity.

Eklund, M. B., Johansson, L. M., Uvnäs-Moberg, K., and Arborelius, L. (2009). Differential effects of repeated long and brief maternal separation on behaviour and neuroendocrine parameters in Wistar dams. *Behav. Brain Res.* 203, 69–75. doi:10.1016/j.bbr.2009.04.017.

Engineer, N., Darwin, L., Nishigandh, D., Ngianga-Bakwin, K., Smith, S. C., and Grammatopoulos, D. K. (2013). Association of glucocorticoid and type 1 corticotropin-releasing hormone receptors gene variants and risk for depression during pregnancy and post-partum. *J. Psychiatr. Res.* 47, 1166–1173. doi:10.1016/j.jpsychires.2013.05.003.

Ennaceur, A., Michalikova, S., van Rensburg, R., and Chazot, P. L. (2010). Tolerance, sensitization and dependence to diazepam in Balb/c mice exposed to a novel open space anxiety test. *Behav. Brain Res.* 209, 154–164. doi:10.1016/j.bbr.2010.01.039.

Evins, G. G., Theofrastous, J. P., and Galvin, S. L. (2000). Postpartum depression: A comparison of screening and routine clinical evaluation. *Am. J. Obstet. Gynecol.* 182, 1080–1082. doi:10.1067/mob.2000.105409.

Falah-Hassani, K., Shiri, R., and Dennis, C.-L. (2016). Prevalence and risk factors for comorbid postpartum depressive symptomatology and anxiety. *J. Affect. Disord.* 198, 142–147. doi:10.1016/j.jad.2016.03.010.

Faraji, J., Karimi, M., Soltanpour, N., Rouhzadeh, Z., Roudaki, S., Hosseini, S. A., et al. (2018). Intergenerational sex-specific transmission of maternal social experience. *Sci. Rep.* 8, 1–14. doi:10.1038/s41598-018-28729-8.

Field, T. (2010). Postpartum Depression Effects on Early Interactions, Parenting, and Safety Practises: a review. 33, 1–9. doi:10.1016/j.infbeh.2009.10.005.Postpartum.

Fitzgerald, P., and Dinan, T. G. (2008). *Prolactin and dopamine: What is the connection? A Review Article*. doi:10.1177/0269216307087148.

Fitzgibbon, M., Finn, D. P., and Roche, M. (2016). High times for painful blues: the endocannabinoid system in pain-depression comorbidity. *Int. J. Neuropsychopharmacol.* 19.

Fleming, A. S., Steiner, M., and Corter, C. (1997). Cortisol, hedonics, and maternal responsiveness in human mothers. *Horm. Behav.* doi:10.1006/hbeh.1997.1407.

Fodor, A., and Zelena, D. (2014). The Effect of Maternal Stress Activation on the Offspring during Lactation in Light of Vasopressin. *Sci. World J.* 2014, 1–15. doi:10.1155/2014/265394.

Frokjaer, V. G., Pinborg, A., Holst, K. K., Overgaard, A., Henningsson, S., Heede, M., et al. (2015). Role of serotonin transporter changes in depressive responses to sex-steroid hormone manipulation: A positron emission tomography study. *Biol. Psychiatry* 78, 534–543. doi:10.1016/j.biopsych.2015.04.015.

Frye, C. A., Petralia, S. M., and Rhodes, M. E. (2000). Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3alpha,5alpha-THP. *Pharmacol. Biochem. Behav.* 67, 587–596.

Fuentes, I. M., Walker, N. K., Pierce, A. N., Holt, B. R., Di Silvestro, E. R., and Christianson, J. A. (2016). Neonatal maternal separation increases susceptibility to experimental colitis and acute stress exposure in male mice. *IBRO Rep.* 1, 10–18. doi:10.1016/j.ibror.2016.07.001.

Gale, S., and Harlow, B. L. (2003). Postpartum mood disorders: A review of clinical and epidemiological factors. *J. Psychosom. Obstet. Gynecol.* 24, 257–266. doi:10.3109/01674820309074690.

Galea, L. A. M., Spritzer, M. D., Barker, J. M., and Pawluski, J. L. (2006). Gonadal hormone modulation of hippocampal neurogenesis in the adult. *Hippocampus* 16, 225–232. doi:10.1002/hipo.20154.

Glynn, L. M., Davis, E. P., and Sandman, C. A. (2013). New insights into the role of perinatal HPA-axis dysregulation in postpartum depression. *Neuropeptides* 47, 363–370. doi:10.1016/j.npep.2013.10.007.

Groer, M. W., and Morgan, K. (2007). Immune, health and endocrine characteristics of depressed postpartum mothers. *Psychoneuroendocrinology*. doi:10.1016/j.psyneuen.2006.11.007.

Gruhn, M. A., Dunbar, J. P., Watson, K. H., Reising, M. M., McKee, L., Forehand, R., et al. (2016). Testing specificity among parents' depressive symptoms, parenting, and child internalizing and externalizing symptoms. *J. Fam. Psychol. JFP J. Div. Fam. Psychol. Am. Psychol. Assoc. Div. 43* 30, 309–319. doi:10.1037/fam0000183.

Guintivano, J., Arad, M., Gould, T. D., Payne, J. L., and Kaminsky, Z. A. (2014). Antenatal prediction of postpartum depression with blood DNA methylation biomarkers. *Mol. Psychiatry* 19, 560–567. doi:10.1038/mp.2013.62.

Heidrich, A., Schleyer, M., Spingler, H., Albert, P., Knoche, M., Fritze, J., et al. (1994). Postpartum blues: Relationship between not-protein bound steroid hormones in plasma and postpartum mood changes. *J. Affect. Disord.* 30, 93–98. doi:10.1016/0165-0327(94)90036-1.

Heilig, M. (2004). The NPY system in stress, anxiety and depression. *Neuropeptides* 38, 213–224. doi:10.1016/j.npep.2004.05.002.

Heim, C., Newport, D. J., Mletzko, T., Miller, A. H., and Nemeroff, C. B. (2008). The link between childhood trauma and depression: Insights from HPA axis studies in humans. *Psychoneuroendocrinology*. doi:10.1016/j.psyneuen.2008.03.008.

Heldring, N., Pike, A., Andersson, S., Matthews, J., Cheng, G., Hartman, J., et al. (2007). Estrogen Receptors: How Do They Signal and What Are Their Targets. *Physiol. Rev.* doi:10.1152/physrev.00026.2006.

Hernández-González, M., Navarro-Meza, M., Prieto-Beracoechea, C. A., and Guevara, M. A. (2005). Electrical activity of prefrontal cortex and ventral tegmental area during rat maternal behavior. *Behav. Processes*. doi:10.1016/j.beproc.2005.06.002.

Huang, W. J., Chen, W. W., and Zhang, X. (2016). Endocannabinoid system: Role in depression, reward and pain control (Review). *Mol. Med. Rep.* 14, 2899–2903. doi:10.3892/mmr.2016.5585.

Jacobs, E., and D'Esposito, M. (2011). Estrogen Shapes Dopamine-Dependent Cognitive Processes: Implications for Women's Health. *J. Neurosci.* doi:10.1523/JNEUROSCI.6394-10.2011.

Janssen, A. B., Kertes, D. A., McNamara, G. I., Braithwaite, E. C., Creeth, H. D. J., Glover, V. I., et al. (2016). *A Role for the Placenta in Programming Maternal Mood and Childhood Behavioural Disorders*. doi:10.1111/jne.12373.

Jones, E., and Coast, E. (2013). Social relationships and postpartum depression in South Asia: A systematic review. *Int. J. Soc. Psychiatry* 59, 690–700. doi:10.1177/0020764012453675.

Josefsson, A., Larsson, C., Sydsjö, G., and Nylander, P. O. (2007). Temperament and character in women with postpartum depression. *Arch. Womens Ment. Health*. doi:10.1007/s00737-006-0159-3.

Kalinichev, M., Easterling, K. W., and Holtzman, S. G. (2000). Periodic postpartum separation from the offspring results in long-lasting changes in anxiety-related behaviors and sensitivity to morphine in Long-Evans mother rats. *Psychopharmacology (Berl.)* 152, 431–439. doi:10.1007/s002130000556.

Kanyicska, L. A., Lerant, A., Freeman, M. E., and Marc, E. (2000). Prolactin : Structure , Function , and Regulation of Secretion MOLECULAR BIOLOGY. *October*. doi:10.1016/B978-0-12-515400-0.50037-3.

Katz, E. R., Stowe, Z. N., Newport, D. J., Kelley, M. E., Pace, T. W., Cubells, J. F., et al. (2012). Regulation of mRNA expression encoding chaperone and co-chaperone proteins of the glucocorticoid receptor in peripheral blood: Association with depressive symptoms during pregnancy. *Psychol. Med.* 42, 943–956. doi:10.1017/S0033291711002121.

Kim, P., Strathearn, L., and Swain, J. E. (2016). The maternal brain and its plasticity in humans. *Horm. Behav.* 77, 113–123. doi:10.1016/j.yhbeh.2015.08.001.

Kim, S., Soeken, T. A., Cromer, S. J., Martinez, S. R., Hardy, L. R., and Strathearn, L. (2014). Oxytocin and postpartum depression: Delivering on what's known and what's not. *Brain Res.* 1580, 219–232. doi:10.1016/j.brainres.2013.11.009.

King, S. M. (1998). Escape-related behaviours in an unstable elevated and exposed environment. *Behav. Brain Res.* 98, 113–126. doi:10.1016/S0166-4328(98)00062-X.

Kinsley, C. H., and Lambert, K. G. (2008). Reproduction-induced neuroplasticity: Natural behavioural and neuronal alterations associated with the production and care of offspring. *J. Neuroendocrinol.* 20, 515–525. doi:10.1111/j.1365-2826.2008.01667.x.

Knox, M., Rosenberger, R., Sarwar, S., Mangewala, V., and Klag, N. (2015). History of postpartum depression and the odds of maternal corporal punishment. *Fam. Syst. Health J. Collab. Fam. Healthc.* 33, 395–399. doi:10.1037/fsh0000157.

Koob, G. F., and Le Moal, M. (1997). Drug abuse: Hedonic homeostatic dysregulation. *Science*. doi:10.1126/science.278.5335.52.

Koob, G. F., and Le Moal, M. (2005). Plasticity of reward neurocircuitry and the'dark side'of drug addiction. *Nat. Neurosci.* 8, 1442.

Korányi, L. (1977). Changes in serum corticosterone and testosterone during induced maternal behavior in rats. *Physiol. Behav.* 18, 287–292.

Korner, A. F. (1973). Early stimulation and maternal care as related to infant capabilities and individual differences†. *Early Child Dev. Care* 2, 307–327. doi:10.1080/0300443730020304.

Kosten, T. A., and Kehoe, P. (2010). Immediate and enduring effects of neonatal isolation on maternal behavior in rats. *Int. J. Dev. Neurosci.* 28, 53–61. doi:10.1016/j.ijdevneu.2009.09.005.

Krishnan, V., and Nestler, E. J. (2011). Animal models of depression: molecular perspectives. *Curr. Top. Behav. Neurosci.* 7, 121–147. doi:10.1007/7854_2010_108.

Larsen, C. M., and Grattan, D. R. (2012). *Prolactin, neurogenesis, and maternal behaviors*. doi:10.1016/j.bbi.2011.07.233.

Leckman, J. F., and Herman, A. E. (2002). Maternal behavior and developmental psychopathology. *Biol. Psychiatry*. doi:10.1016/S0006-3223(01)01277-X.

Lee, R. S. C., Hermens, D. F., Porter, M. A., and Redoblado-Hodge, M. A. (2012). *A meta-analysis of cognitive deficits in first-episode Major Depressive Disorder*. doi:10.1016/j.jad.2011.10.023.

Lehmann, J., and Feldon, J. (2000). Long-term biobehavioral effects of maternal separation in the rat: Consistent or confusing? *Rev. Neurosci.* 11, 383–408. doi:10.1515/REVNEURO.2000.11.4.383.

Leo, L., and Pamplona, F. (2014). Elevated Plus Maze Test to Assess Anxiety-like Behavior in the Mouse. *BIO-Protoc.* 4. doi:10.21769/BioProtoc.1211.

Letourneau, N. L., Dennis, C. L., Cosic, N., and Linder, J. (2017). The effect of perinatal depression treatment for mothers on parenting and child development: A systematic review. *Depress. Anxiety* 34, 928–966. doi:10.1002/da.22687.

Leuner, B., Glasper, E. R., and Gould, E. (2010). *Parenting and plasticity*. doi:10.1016/j.tins.2010.07.003.

Leuner, B., and Shors, T. J. (2006). Learning during motherhood: A resistance to stress. *Horm. Behav.* 50, 38–51. doi:10.1016/j.yhbeh.2006.01.002.

Leuner, Mirescu, C., Noiman, L., and Elizabeth Gould (2007). Maternal Experience Inhibits the Production of Immature Neurons in the Hippocampus During the Postpartum Period Through Elevations in Adrenal Steroids. *Hippocampus* 37, 36–41. doi:10.1002/hipo.

Leussis, M. P., and Bolivar, V. J. (2006). Habituation in rodents: a review of behavior, neurobiology, and genetics. *Neurosci. Biobehav. Rev.* 30, 1045–1064. doi:10.1016/j.neubiorev.2006.03.006.

Levine, S. (2000). Influence of psychological variables on the activity of the hypothalamic-pituitary-adrenal axis. *Eur. J. Pharmacol.* 405, 149–160. doi:10.1016/S0014-2999(00)00548-3.

Levine, S. (2005). Developmental determinants of sensitivity and resistance to stress. *Psychoneuroendocrinology* 30, 939–946. doi:10.1016/j.psyneuen.2005.03.013.

Li, M., and Chou, S.-Y. (2016). Modeling postpartum depression in rats: theoretic and methodological issues. *Zool. Res.* doi:10.13918/j.issn.2095-8137.2016.4.229.

Lovejoy, M. C., and Graczyk, P. A. (2000). Maternal depression and parenting behavior: A meta-analytic review. *Technology* 44, 0–8. doi:10.1016/S0272-7358(98)00100-7.

Mackie, K. (2005). Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb. Exp. Pharmacol.* doi:10.1007/3-540-26573-2-10.

Macrì, S., Chiarotti, F., and Würbel, H. (2008). Maternal separation and maternal care act independently on the development of HPA responses in male rats. *Behav. Brain Res.* 191, 227–234. doi:10.1016/j.bbr.2008.03.031.

Magiakou, M.-A., Mastorakos, G., Rabin, D., Dubbert, B., Gold, P. W., and Chrousos, G. P. (1996). the Postpartum Period : Implications for the Increase Manifestations. *J. Clin. Endocrinol. Metab.* 81, 1912–1917.

Mahmoud, J. S. R., Staten, R. "Topsy," Hall, L. A., and Lennie, T. A. (2012). The Relationship among Young Adult College Students' Depression, Anxiety, Stress, Demographics, Life Satisfaction, and Coping Styles. *Issues Ment. Health Nurs.* 33, 149–156. doi:10.3109/01612840.2011.632708.

Maier, S. F., and Seligman, M. E. P. (2016). Learned helplessness at fifty: Insights from neuroscience. *Psychol. Rev.* 123, 349–367. doi:10.1037/rev0000033.

Maniam, J., and Morris, M. J. (2010). Long-term postpartum anxiety and depression-like behavior in mother rats subjected to maternal separation are ameliorated by palatable high fat diet. *Behav. Brain Res.* doi:10.1016/j.bbr.2009.11.005.

Manzanares, J., Corchero, J., Romero, J., Fernández-Ruiz, J. J., Ramos, J. A., and Fuentes, J. A. (1999). Pharmacological and biochemical interactions between opioids and cannabinoids. *Trends Pharmacol. Sci.* 20, 287–294. doi:10.1016/S0165-6147(99)01339-5.

Matthey, S., Barnett, B., Howie, P., and Kavanagh, D. J. (2003). Diagnosing postpartum depression in mothers and fathers: Whatever happened to anxiety? *J. Affect. Disord.* doi:10.1016/S0165-0327(02)00012-5.

Maurer-Spurej, E., Pittendreigh, C., and Misri, S. (2007). Platelet serotonin levels support depression scores for women with postpartum depression. *J. Psychiatry Neurosci.* 32, 23–29.

Mayer, A. D., Ahdieh, H. B., and Rosenblatt, J. S. (1990). Effects of prolonged estrogen-progesterone treatment and hypophysectomy on the stimulation of short-latency maternal behavior and aggression in female rats. *Horm. Behav.* 24, 152–173. doi:10.1016/0018-506X(90)90002-F.

McCormick, C. M., and Mathews, I. Z. (2007). *HPA function in adolescence: Role of sex hormones in its regulation and the enduring consequences of exposure to stressors*. doi:10.1016/j.pbb.2006.07.012.

McEwen (1998). Stress, Adaptation, and Disease: Allostasis and Allostatic Load. *Ann. N. Y. Acad. Sci.* doi:10.5958/0976-0571.2014.00691.2.

McIntosh, J., Anisman, H., and Merali, Z. (1999). Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: Gender-dependent effects. *Dev. Brain Res.* 113, 97–106. doi:10.1016/S0165-3806(99)00005-X.

Mechan, A. O., Moran, P. M., Elliott, M., Young, A. J., Joseph, M. H., and Green, R. (2002). A comparison between Dark Agouti and Sprague-Dawley rats in their behaviour on the elevated plus-maze, open-field apparatus and activity meters, and their response to diazepam. *Psychopharmacology (Berl.)* 159, 188–195. doi:10.1007/s002130100902.

Michaels, C. C., and Holtzman, S. G. (2006). Neonatal stress and litter composition alter sucrose intake in both rat dam and offspring. *Physiol. Behav.* 89, 735–741. doi:10.1016/j.physbeh.2006.08.015.

Michaels, C. C., and Holtzmann, S. G. (2007). Enhanced Sensitivity to Naltrexone-induced Drinking Suppression of Fluid Intake and Sucrose Consumption in Maternally Separated Rats. 64, 2391–2404. doi:10.1038/jid.2014.371.

Milgrom, J., Schembri, C., Ericksen, J., Ross, J., and Gemmill, A. W. (2011). Towards parenthood: An antenatal intervention to reduce depression, anxiety and parenting difficulties. *J. Affect. Disord.* 130, 385–394. doi:10.1016/j.jad.2010.10.045.

Millstein, R. A., and Holmes, A. (2007). Effects of repeated maternal separation on anxiety- and depression-related phenotypes in different mouse strains. *Neurosci. Biobehav. Rev.* 31, 3–17. doi:10.1016/j.neubiorev.2006.05.003.

Moffett, M. C., Harley, J., Francis, D., Sanghani, S. P., Davis, W. I., and Kuhar, M. J. (2006). Maternal Separation and Handling Affects Cocaine Self- Administration in Both the Treated Pups as Adults and the Dams. *Pharmacology* 317, 1210–1218. doi:10.1124/jpet.106.101139.

Molendijk, M. L., and de Kloet, E. R. (2015). Immobility in the forced swim test is adaptive and does not reflect depression. *Psychoneuroendocrinology* 62, 389–391.

Monleon, S., Parra, A., Simon, V. M., Brain, P. F., D'Aquila, P., and Willner, P. (1995). Attenuation of sucrose consumption in mice by chronic mild stress and its restoration by imipramine. *Psychopharmacology (Berl.)*. doi:10.1007/BF02246218.

Monteiro, S., Roque, S., de Sá-Calçada, D., Sousa, N., Correia-Neves, M., and Cerqueira, J. J. (2015). An efficient chronic unpredictable stress protocol to induce stress-related responses in C57BL/6 mice. *Front. Psychiatry* 6, 6. doi:10.3389/fpsyt.2015.00006.

Moore, C. (1982). Maternal behavior of rats is affected by hormonal condition of pups. *J. Comp. Physiol. Psychol.* 96, 123–129. doi:10.1037/h0077866.

Moses-Kolko, E. L. (2010). Abnormally Reduced Dorsomedial Prefrontal Cortical Activity and Effective Connectivity With Amygdala in Response to Negative Emotional Faces in Postpartum Depression Eydie. *Am J Psychiatry* 16711, 1373–1380.

Moses-Kolko, E. L., Fraser, D., Wisner, K. L., James, J. A., Saul, A. T., Fiez, J. A., et al. (2011). Rapid habituation of ventral striatal response to reward receipt in postpartum depression. *Biol. Psychiatry* 70, 395–399. doi:10.1016/j.biopsych.2011.02.021.

Moses-Kolko, E. L., Price, J. C., Wisner, K. L., Hanusa, B. H., Meltzer, C. C., Berga, S. L., et al. (2012). Postpartum and depression status are associated with lower 11 craclopride BP ND in reproductive-age women. *Neuropsychopharmacology* 37, 1422–1432. doi:10.1038/npp.2011.328.

Moses-Kolko, E. L., Wisner, K. L., Price, J. C., Berga, S. L., Drevets, W. C., Hanusa, B. H., et al. (2008). Serotonin 1A receptor reductions in postpartum depression: a positron emission tomography study. *Fertil. Steril.* 89, 685–692. doi:10.1016/j.fertnstert.2007.03.059.

Möstl, E., and Palme, R. (2002). Hormones as indicators of stress. in *Domestic Animal Endocrinology* doi:10.1016/S0739-7240(02)00146-7.

Mouslech, Z., and Valla, V. (2009). Endocannabinoid system: An overview of its potential in current medical practice. *Neuro Endocrinol. Lett.* 30, 153–179.

Murray, L., and Cooper, P. J. (2013). EDITORIAL : Postpartum depression and child development. 253–260. doi:10.1017/S0033291796004564.

Nagy, G., Banky, Z., and Takacs, L. (1983). Rapid decrease of plasma prolactin levels in lactating rats separated from their pups. *Endocrinol. Exp.*

Nasehi, M., Sharifi, S., and Zarrindast, M. R. (2012). Involvement of the cholinergic system of CA1 on harmane-induced amnesia in the step-down passive avoidance test. *J. Psychopharmacol. (Oxf.)*. doi:10.1177/0269881111421972.

Nelson, E. N. (1998). Brain Substrates of Infant–Mother Attachment: Contributions of Opioids, Oxytocin, and Norepinephrine. *Descr. Transl. Stud. Investig. Homage Gideon Toury* 22, 209–221. doi:10.1016/S0149-7634(97)00052-3.

Netto, C. A., and Izquierdo, I. (1985). On how passive is inhibitory avoidance. *Behav. Neural Biol.* doi:10.1016/S0163-1047(85)91697-8.

Nielsen, D., Videbech, P., Hedegaard, M., Dalby, J., and Secher, N. J. (2000). Postpartum depression: Identification of women at risk. *Br. J. Obstet. Gynaecol.* 107, 1210–1217. doi:10.1111/j.1471-0528.2000.tb11609.x.

Nofrey, B., Rocha, B., Lopez, H. H., and Ettenberg, A. (2008). The effects of sexual experience and estrus on male-seeking motivated behavior in the female rat. *Physiol. Behav.* doi:10.1016/j.physbeh.2008.08.002.

Numan, M., and Stolzenberg, D. S. (2009). *Medial preoptic area interactions with dopamine neural systems in the control of the onset and maintenance of maternal behavior in rats*. doi:10.1016/j.yfrne.2008.10.002.

O'Hara, M., and McCabe, J. (2013). Postpartum Depression: Current Status and Future Directions. *Ssrn*. doi:10.1146/annurev-clinpsy-050212-185612.

Öhman, A. (2005). *The role of the amygdala in human fear: Automatic detection of threat*. doi:10.1016/j.psyneuen.2005.03.019.

Olza-Fernández, I., Marín Gabriel, M. A., Gil-Sanchez, A., Garcia-Segura, L. M., and Arevalo, M. A. (2014). *Neuroendocrinology of childbirth and mother-child attachment: The basis of an etiopathogenic model of perinatal neurobiological disorders*. doi:10.1016/j.yfrne.2014.03.007.

O'Malley, J., Dambrosia, J. M., and Davis, J. a (2008). Effect of housing density on reproductive parameters and corticosterone levels in nursing mice. *J. Am. Assoc. Lab. Anim. Sci. JAALAS*.

Opendak, M., Briones, B. A., and Gould, E. (2016). Social behavior, hormones and adult neurogenesis. *Front. Neuroendocrinol.* 41, 71–86. doi:10.1016/j.yfrne.2016.02.002.

Orpen, B. G., and Fleming, A. S. (1987). Experience with pups sustains maternal responding in postpartum rats. *Physiol. Behav.* 40, 47–54. doi:10.1016/0031-9384(87)90184-3.

Orso, R., Creutzberg, K. C., Wearick-Silva, L. E., Wendt Viola, T., Tractenberg, S. G., Benetti, F., et al. (2019). How Early Life Stress Impact Maternal Care: A Systematic Review of Rodent Studies. *Front. Behav. Neurosci.* 13. doi:10.3389/fnbeh.2019.00197.

Orso, R., Wearick-Silva, L. E., Creutzberg, K. C., Centeno-Silva, A., Glusman Roithmann, L., Pazzin, R., et al. (2018). Maternal behavior of the mouse dam toward pups: implications for maternal separation model of early life stress. *Stress*. doi:10.1080/10253890.2017.1389883.

Papp, M., Willner, P., and Muscat, R. (1991). An animal model of anhedonia: attenuation of sucrose consumption and place preference conditioning by chronic unpredictable mild stress. *Psychopharmacology (Berl.)*. doi:10.1007/BF02244188.

Pariante, C. M., and Lightman, S. L. (2008). *The HPA axis in major depression: classical theories and new developments*. doi:10.1016/j.tins.2008.06.006.

Patel, S., Hill, M. N., Cheer, J. F., Wotjak, C. T., and Holmes, A. (2017). The endocannabinoid system as a target for novel anxiolytic drugs. *Neurosci. Biobehav. Rev.* 76, 56–66. doi:10.1016/j.neubiorev.2016.12.033.

Paulson, J. F., Dauber, S., and Leiferman, J. A. (2006). Individual and Combined Effects of Postpartum Depression in Mothers and Fathers on Parenting Behavior. *PEDIATRICS* 118, 659–668. doi:10.1542/peds.2005-2948.

Pawluski, J. L., Lonstein, J. S., and Fleming, A. S. (2017). The Neurobiology of Postpartum Anxiety and Depression. *Trends Neurosci.* 40, 106–120. doi:10.1016/j.tins.2016.11.009.

Pedersen, C. A., Caldwell, J. D., Walker, C., Ayers, G., and Mason, G. A. (1994). Oxytocin activates the postpartum onset of rat maternal behavior in the ventral tegmental and medial preoptic areas. *Behav. Neurosci.* doi:10.1037/0735-7044.108.6.1163.

Pellow, S., Chopin, P., File, S. E., and Briley, M. (1985). Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods*. doi:10.1016/0165-0270(85)90031-7.

Pellow, S., and File, S. E. (1986). Anxiolytic and anxiogenic drug effects on exploratory activity in an elevated plus-maze: A novel test of anxiety in the rat. *Pharmacol. Biochem. Behav.* doi:10.1016/0091-3057(86)90552-6.

Philipps, L. H., and O'Hara, M. W. (1991). Prospective study of postpartum depression: 4½-year follow-up of women and children. *J. Abnorm. Psychol.* 100, 151–155. doi:10.1037/0021-843X.100.2.151.

Picazo, O., Rosenblatt, J. S., and Fernández-Guasti, A. (2000). The differential effect of the anxiolytic agent 8-OH-DPAT during lactation is independent of pup withdrawal and maternal behavior. *Psychoneuroendocrinology* 25, 693–706. doi:10.1016/S0306-4530(00)00019-6.

Pizzagalli, D. A., Iosifescu, D., Hallett, L. A., Ratner, K. G., and Fava, M. (2008). Reduced hedonic capacity in major depressive disorder: evidence from a probabilistic reward task. *J. Psychiatr. Res.* 43, 76–87.

Pryce, C. R., Bettschen, D., and Feldon, J. (2001). Comparison of the effects of early handling and early deprivation on maternal care in the rat. *Dev. Psychobiol.* 38, 239–251. doi:10.1002/dev.1018.

Rasia-Filho, A. A., Fabian, C., Rigoti, K. M., and Achaval, M. (2004). Influence of sex, estrous cycle and motherhood on dendritic spine density in the rat medial amygdala revealed by the Golgi method. *Neuroscience* 126, 839–847. doi:10.1016/j.neuroscience.2004.04.009.

Rasia-Filho, A. A., Londero, R. G., and Achaval, M. (2000). Functional activities of the amygdala: An overview. *J. Psychiatry Neurosci.* 25, 14–23.

Reck, C., Struben, K., Backenstrass, M., Stefenelli, U., Reinig, K., Fuchs, T., et al. (2008). Prevalence, onset and comorbidity of postpartum anxiety and depressive disorders. *Acta Psychiatr. Scand.* doi:10.1111/j.1600-0447.2008.01264.x.

Redrobe, J. P., Dumont, Y., and Quirion, R. (2002). Neuropeptide Y (NPY) and depression: From animal studies to the human condition. *Life Sci.* 71, 2921–2937. doi:10.1016/S0024-3205(02)02159-8.

Rincón-Cortés, M., and Sullivan, R. M. (2014). Early Life Trauma and Attachment: Immediate and Enduring Effects on Neurobehavioral and Stress Axis Development. *Front. Endocrinol.* 5. doi:10.3389/fendo.2014.00033.

Robertson, E., Grace, S., Wallington, T., and Stewart, D. E. (2004). Antenatal risk factors for postpartum depression: A synthesis of recent literature. *Gen. Hosp. Psychiatry* 26, 289–295. doi:10.1016/j.genhosppsych.2004.02.006.

Romeo, R. D., Mueller, A., Sisti, H. M., Ogawa, S., McEwen, B. S., and Brake, W. G. (2003). Anxiety and fear behaviors in adult male and female C57BL/6 mice are modulated by maternal separation. *Horm. Behav.* 43, 561–567. doi:10.1016/S0018-506X(03)00063-1.

Ross, L. E., and McLean, L. M. (2006). Anxiety Disorders During Pregnancy and the Postpartum Period: A Systematic Review. *J. Clin. Psychiatry* 67, 1285–1298. doi:10.4088/JCP.v67n0818.

Russo, S. J., and Nestler, E. J. (2013). The brain reward circuitry in mood disorders. *Nat. Rev. Neurosci.* 14, 609.

Sakaki, M., and Mather, M. (2012). *How reward and emotional stimuli induce different reactions across the menstrual cycle*. doi:10.1111/j.1751-9004.2011.00415.x.

Sarason, I. G. (1984). Stress, anxiety, and cognitive interference: Reactions to tests. *J. Pers. Soc. Psychol.* 46, 929–938. doi:10.1037/0022-3514.46.4.929.

Schiller, C. E., Meltzer-Brody, S., and Rubinow, D. R. (2015). The role of reproductive hormones in postpartum depression. *CNS Spectr.* 20, 48–59. doi:10.1017/S1092852914000480.

Schulte, Weisner, and Allolio (1990). THE CORTICOTROPHIN RELEASING HORMONE TEST IN LATE PREGNANCY: LACK OF ADRENOCORTICOTROPHIN AND CORTISOL RESPONSE. *Clin. Endocrinol. (Oxf.)*. doi:10.1111/j.1365-2265.1990.tb00470.x.

Serati, M., Redaelli, M., Buoli, M., and Altamura, A. C. (2016). Perinatal Major Depression Biomarkers: A systematic review. *J. Affect. Disord.* 193, 391–404. doi:10.1016/j.jad.2016.01.027.

Seth, S., Lewis, A. J., and Galbally, M. (2016). Perinatal maternal depression and cortisol function in pregnancy and the postpartum period: A systematic literature review. *BMC Pregnancy Childbirth* 16. doi:10.1186/s12884-016-0915-y.

Shanmugan, S., and Epperson, C. N. (2014). *Estrogen and the prefrontal cortex: Towards a new understanding of estrogen's effects on executive functions in the menopause transition*. doi:10.1002/hbm.22218.

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R., and Boonstra, R. (2011). *Measuring stress in wildlife: Techniques for quantifying glucocorticoids*. doi:10.1007/s00442-011-1943-y.

Soares, C. N., and Zitek, B. (2008). Reproductive hormone sensitivity and risk for depression across the female life cycle: A continuum of vulnerability? *J. Psychiatry Neurosci.* 33, 331–343.

Stanton, M. E., Gutierrez, Y. R., and Levine, S. (1988). Maternal deprivation potentiates pituitary-adrenal stress responses in infant rats. *Behav. Neurosci.* 102, 692–700. doi:10.1037/0735-7044.102.5.692.

Stewart, D. E. (2013). Postpartum depression. *J. ET Nurs.* 60, 22–26. doi:10.6224/JN.60.6.22.

Sung, Y. H., Shin, M. S., Cho, S., Baik, H. H., Jin, B. K., Chang, H. K., et al. (2010). Depression-like state in maternal rats induced by repeated separation of pups is accompanied by a decrease of cell proliferation and an increase of apoptosis in the hippocampus. *Neurosci. Lett.* 470, 86–90. doi:10.1016/j.neulet.2009.12.063.

Svíženská, I. (2008). Cannabinoid receptors 1 and 2 (CB1 and CB2), their distribution, ligands and functional involvement in nervous system structures — A short review. *Phys. Sportsmed.* 19, 501–511. doi:10.1016/j.pbb.2008.05.010.

Sweep, C. G. J. (Fred) (1989). Beta-endorphin in brain limbic structures as neurochemical correlate of psychic dependence on drugs. *Life Sci.* 44, 1133–1140.

T. Abou-Saleh, M., Ghubash, R., Karim, L., Krymski, M., and Bhai, I. (1998). Hormonal aspects of postpartum depression. *Psychoneuroendocrinology* 23, 465–475. doi:10.1016/S0306-4530(98)00022-5.

Tan, S., Ho, H. S., Song, A. Y., Low, J., and Je, H. S. (2017). Maternal Separation Does Not Produce a Significant Behavioral Change in Mice. *Exp. Neurobiol.* doi:10.5607/en.2017.26.6.390.

Torner, L. (2016). Actions of prolactin in the brain: From physiological adaptations to stress and neurogenesis to psychopathology. *Front. Endocrinol.* 7, 1–6. doi:10.3389/fendo.2016.00025.

Tsuda, M. C., Yamaguchi, N., Nakata, M., and Ogawa, S. (2014). Modification of female and male social behaviors in estrogen receptor beta knockout mice by neonatal maternal separation. *Front. Neurosci.* doi:10.3389/fnins.2014.00274.

Ulrich-Lai, Y. M., Figueiredo, H. F., Ostrander, M. M., Choi, D. C., Engeland, W. C., and Herman, J. P. (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *AJP Endocrinol. Metab.* doi:10.1152/ajpendo.00070.2006.

Vilela, F. C., Vieira, J. S., Giusti-Paiva, A., and Silva, M. L. da (2017). Experiencing early life maternal separation increases pain sensitivity in adult offspring. *Int. J. Dev. Neurosci.* 62, 8–14. doi:10.1016/j.ijdevneu.2017.07.003.

Von Poser Toigo, E., Diehl, L. A., Ferreira, A. G. K., MacKendanz, V., Krolow, R., Benitz, A. N. D., et al. (2012). Maternal depression model: Long-lasting effects on the mother following separation from pups. *Neurochem. Res.* 37, 126–133. doi:10.1007/s11064-011-0590-3.

Wadhwa, P. D., Sandman, C. A., and Garite, T. J. (2001). "Chapter 9 The neurobiology of stress in human pregnancy: implications for prematurity and development of the fetal central nervous system," in *Progress in Brain Research* (Elsevier), 131–142. doi:10.1016/S0079-6123(01)33010-8.

Watkins, S., Meltzer-Brody, S., Zolnoun, D., and Stuebe, A. (2011). Early breastfeeding experiences and postpartum depression. *Obstet. Gynecol.* 118, 214–221. doi:10.1097/AOG.0b013e3182260a2d.

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., et al. (2004). Epigenetic programming by maternal behavior. *Nat. Neurosci.* doi:10.1038/nn1276.

Wei, L., David, A., Duman, R. S., Anisman, H., and Kaffman, A. (2010). Early life stress increases anxiety-like behavior in Balbc mice despite a compensatory increase in levels of postnatal maternal care. *Horm. Behav.* 57, 396–404. doi:10.1016/j.yhbeh.2010.01.007.

Weinberg, M. K., Beeghly, M., Olson, K. L., and Tronick, E. (2008). Effects of maternal depression and panic disorder on mother-infant interactive behavior in the Face-to-Face Still-Face paradigm. *Infant Ment. Health J.* doi:10.1002/imhj.20193.

Weissman, M. M., Klerman, G. L., Paykel, E. S., Prusoff, B., and Hanson, B. (1974). Treatment Effects on the Social Adjustment of Depressed Patients. *Arch. Gen. Psychiatry*. doi:10.1001/archpsyc.1974.01760120033006.

Weissman, M. M., Paykel, E. S., and Klerman, G. L. (1972). The depressed woman as a mother. *Soc. Psychiatry*. doi:10.1007/BF00583985.

Wheelan, N., Kenyon, C. J., Harris, A. P., Cairns, C., Al Dujaili, E., Seckl, J. R., et al. (2018). Midlife stress alters memory and mood-related behaviors in old age: Role of locally activated glucocorticoids. *Psychoneuroendocrinology* 89, 13–22. doi:10.1016/j.psyneuen.2017.12.018.

Williams, M. E., Becker, S., McKinnon, M. C., Wong, Q., Cudney, L. E., Steiner, M., et al. (2015). Emotional memory in pregnant women at risk for postpartum depression. *Psychiatry Res.* 229, 777–783. doi:10.1016/j.psychres.2015.08.003.

Wong, D. F., Kuwabara, H., Horti, A. G., Raymont, V., Brasic, J., Guevara, M., et al. (2010). Quantification of cerebral cannabinoid receptors subtype 1 (CB1) in healthy subjects and schizophrenia by the novel PET radioligand [11C]OMAR. *NeuroImage* 52, 1505–1513. doi:10.1016/j.neuroimage.2010.04.034.

Xie, R., Xie, H., Daniel, K., and He, G. (2018). Plasma concentrations of neurotransmitters and postpartum depression. *J. Cent. South Univ. Med. Sci.* 43, 274–281. doi:10.11817/j.issn.1672-7347.2018.03.007.

Yankelevitch-Yahav, R., Franko, M., Huly, A., and Doron, R. (2015). The forced swim test as a model of depressive-like behavior. *J. Vis. Exp. JoVE*. doi:10.3791/52587.

Yim, I. S., Glynn, L. M., Schetter, C. D., Hobel, C. J., Chicz-Demet, A., and Sandman, C. A. (2010). Prenatal β-endorphin as an early predictor of postpartum depressive symptoms in euthymic women. *J. Affect. Disord.* 125, 128–133. doi:10.1016/j.jad.2009.12.009.

Zarrow, M. X., Schlein, P. A., Denenberg, V. H., and Cohen, H. A. (1972). Sustained corticosterone release in lactating rats following olfactory stimulation from the pups. *Endocrinology* 91, 191–196. doi:10.1210/endo-91-1-191.

Zimmerberg, B., Rosenthal, A. J., and Stark, A. C. (2003). Neonatal social isolation alters both maternal and pup behaviors in rats. *Dev. Psychobiol.* 42, 52–63. doi:10.1002/dev.10086.

Maternal separation in rat dams – a neurobiological and behavioral approach to characterize the maternal side

Ibrahim Bölükbas[1], Annakarina Mundorf [1] and Nadja Freund[1]

[1] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum

Research article / submitted

Abstract

The time after parturition is a sensitive period for mothers where they are prone to develop psychopathological symptoms. Studies investigating dams after maternal separation (MS) showed that MS induces alterations similar to postpartum depression. This study aims to give further details of affected behavior and neurobiology of dams after MS.

MS from postnatal day 2 to 20 over four hours daily was performed. Upon reunion, maternal care and ultrasonic vocalization of dams were measured. On day of weaning, dams were tested for anxiety-like behavior in the elevated-plus-maze and marble burying test. Then *Morc1* mRNA in the medial prefrontal cortex and *Nr3c1* mRNA in the hippocampus were measured using real-time PCR. GABA and glutamate serum levels were analyzed by high-performance liquid chromatography.

MS in dam's increased maternal care towards pups and 50-kHz and 22-kHz emission. No differences in anxiety-like behavior were detected. MS further reduced *Morc1* but not *Nr3c1* expression. Serum GABA but not glutamate levels were significantly increased in separated dams.

This study reinforces the benefit of investigating dams after MS for studying postpartum stress. Subclinical markers mainly connected to depression, namely *Morc1* and GABA, proved to be useful allowing for earlier detection of symptoms of critical postpartum stress.

Keywords: *Morc1*, *Nr3c1*, maternal behavior, USV, postpartum, stress, GABA, Glutamate

1. Introduction

The maternal separation (MS) paradigm is a recognized animal model to study early life stress (ELS) [1–3]. MS involves repeated daily separations of the dams from their litters [4–6] which induces long-lasting behavioral and neurobiological changes in the offspring [7–9]. While the stress exposure in this sensitive phase of early development has been widely studied, it is often ignored that the postpartum phase is also a vulnerable phase for dams or mothers making them prone to develop psychiatric disorders e.g. postpartum depression (PPD) [10].

Only a few studies have so far focused on consequences for the dams experiencing MS [11,12]. These studies mostly investigated influences of repeated MS on maternal behavior measuring parameters such as time licking and nursing the pups or arched-back nursing[13] and anxiety- or depression-like behavior showing an increase of both, maternal care as well as anxiety- and depression-like behavior after MS [14]. Anxiety-like behavior was measured using elevated plus maze (EPM) with most studies showing decreased time spent in the open arms implying increased anxiety[12,15]. Increased depressive-like behavior was induced and demonstrated using forced-swim test with increased immobility time [16,17] or less sucrose intake in the sucrose consumption test [12]. Furthermore, ultrasonic vocalization (USV) emitted by the dams upon reunion can be detected as an established tool to assess affective state [18]. In rats, 50-kHz frequencies are associated with positive affect whereas 22-kHz frequencies are often reported being emitted in aversive situations such as anxiety [19]. Mother-infant disruptions have shown to induce vocalization alterations with more 50-kHz frequencies emitted only after pups were returned [18]. Another study revealed that chronic mother-infant separation has increased the number of USVs in pups [20].

The neuronal mechanisms that are affected by the separation and induce behavioral changes are largely unknown. Studies investigating dams after MS have found lower *glucocorticoid-receptor* (GR) mRNA in hippocampus and elevated plasma CORT levels of confirming the stress reaction induced by separation from the pups [12]. These studies also found that imbalances in hormones and neurotransmitters are similar to those observed in humans suffering from PPD such as altered cortisol, prolactin levels or differences in the serotonin-system [1216,21].

The connection between the separation, elevated maternal care, stress and development of depressive-like symptoms is unclear but might provide valuable insights for postpartum depression in humans.

The *MORC Family CW-Type Zinc Finger 1* (*Morc1*) gene has recently been connected to stress experience during sensitive phases (so far ELS) and Depression [22–24]. Besides human

studies, investigating *Morc1* knockout mice revealed depressive-like behavior without any other behavioral deficits [25].

Morc1's role in ELS has been discovered in downregulation of *Morc1* in the medial prefrontal cortex (mPFC) [22] and using a genome-wide association study data set has been linked to Major Depressive Disorder (MDD) [22]. Investigating DNA methylation of MORC1 in healthy controls as well as in patients showed significant associations between methylation status and depressive symptoms [23,24]. Now it would be interesting to investigate whether *Morc1* could also serve as an indicator for stress exposure in the postpartum phase, especially as *Morc1* has proven to be a reliable indicator for subclinical depressive symptoms as well [23]. As a key region in cognitive behavior, personality expression and moderating social behavior, the medial prefrontal cortex (mPFC) will be analyzed regarding *Morc1* expression.

On peripheral level, two key mood regulating neurotransmitters such as glutamate and gamma-Aminobutyric acid (GABA) are getting broader attention in the pathogenesis of stress-related disorders [26–29]. MS has demonstrated to permanently alter GABAergic and glutamate transmission and behavioral stress responses [30,31]. Several clinical studies demonstrate altered concentrations of glutamate [32] and GABA [33] in depressed patients' serum, plasma or cerebrospinal fluids. Thus, investigating glutamate and GABA serum levels in dams after MS could provide further insights but also enable translational results comparable with findings in humans. In this study, we combine a behavioral, neurochemical and peripheral approach to characterize dams' alterations.

2. Results

2.1. Behavioral Results

2.1.1. Maternal care and USV detection

Repeated measurements for parameters over the 4 testing days showed significant results between the groups: $F_{(1/15)}$=286.25; P=0.000.

Dams subjected to separation exhibited significantly more licking and grooming (LG) than non-separated dams at all days measured (PD2: $t_{(14)}$ = -2.875; P = .01; PD6: $t_{(14)}$ = -3,96; P = .00; PD14: $t_{(14)}$ = -4,62; P = .00; PD20: $t_{(14)}$ = -4,72; P = .00, **FIG.1A**).

Arched-back nursing also significantly differed at the 4 time points with separated dams displaying more arched-back nursing behavior (PD2: $t_{(14)}$ = -4,537; P = .00; PD6: $t_{(14)}$ = -8,68; P = .00; PD14: $t_{(14)}$ = -4,537; P = .00; PD20: $t_{(14)}$ = -5.9; P = .00; see **FIG. 1B**).

Self-grooming behavior was significantly lower in separated dams at PD6, PD14 and PD20, but not at PD2 (PD2: $t_{(14)}$ = 2.08; P = .06; PD6: $t_{(14)}$ = 3.19; P = .01; PD14: $t_{(14)}$ = 3,73; P = .00; PD20: $t_{(14)}$ = 6,26, P = .00). Interestingly, the rearing time also significantly differed at

PD6 and PD20 with lower scores in separated dams (PD6: $t_{(14)}$ = 3,16, P = .00; PD20: $t_{(14)}$ = 3,21; P = .01).

Repeated measures for 50-kHz frequencies with unmodulated flat frequencies for all days measured revealed significant differences between days ($F_{(1/14)}$ = 121.37; P=0.000), see **Fig. 1C**. The 22-kHz frequencies between groups also showed significant results when all 4 days measured ($F_{(1/15)}$=13.64; P =0.002), see **Fig. 1D.** For both, 22- and 50-kHz significant results were obtained at PD20 but not in the other days when using student's t-test (for 50-kHz PD20: $t_{(14)}$ = -3,957, P = 0.00; for 22-kHz PD20: control: $t_{(14)}$ = -2,65; P = 0.02).

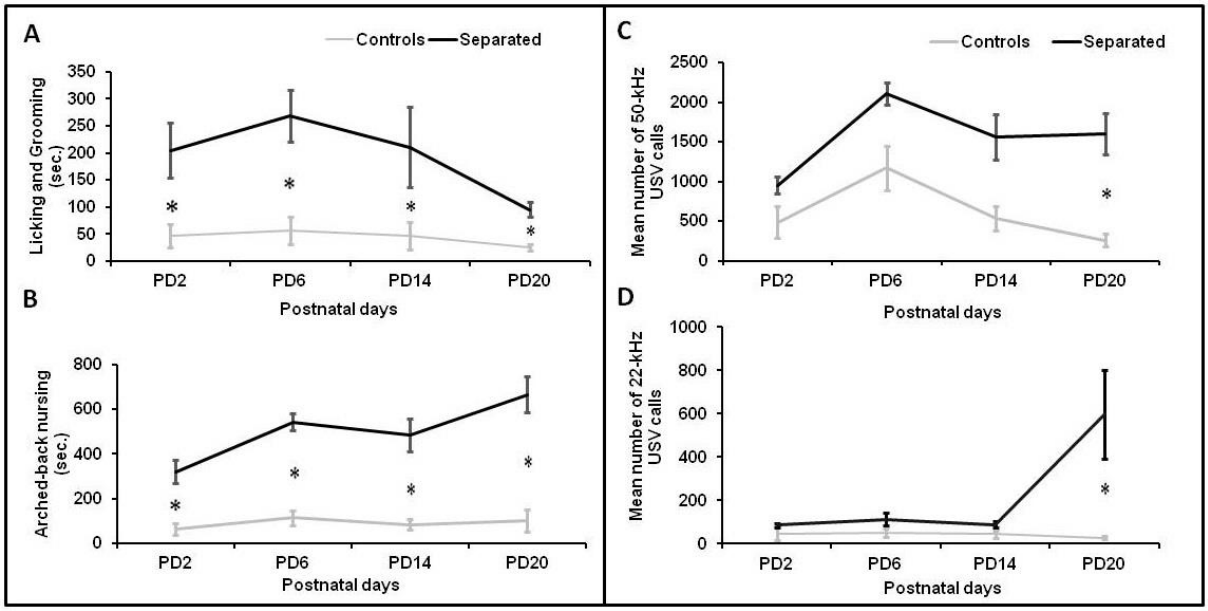


Fig.1A: Licking and grooming behavior at PD2, PD6, PD14 and PD20 in separated (black) and unseparated (grey) dams. Separated dams were expressing significantly higher licking and grooming behavior. Means ± SEM are presented for n = 8 controls and n = 8 separated dams. Data was analyzed by student's t-test.

Fig.1B: Arched-back nursing behavior at PD2, PD6, PD14 and PD20 in separated (black) and unseparated (grey) dams. Separated dams were spending significantly higher time in arched-back nursing position. Means ± SEM are presented for n = 8 controls and n = 8 separated dams. Data was analyzed by student's t-test.

Fig 1C: Number of 50-kHz frequencies: Separation dams (black) vocalized significantly more at 50-kHz frequencies at the last day measured compared to control dams (grey). Means ± SEM are presented for n = 8 controls and n = 8 separated dams. Data was analyzed by student's t-test.

Fig 1D: Number of 22-kHz frequencies showing that separation dams (black) vocalized significantly more at 22-kHz frequencies at the last day measured compared to control dams (grey). Means ± SEM are presented for n = 8 controls and n = 8 MS. Data was analyzed by student's t-test.

2.1.2. Anxiety-like behavior

EPM did not reveal significant differences between groups by time spent in the open arms (control: $t_{(10)}$ = 1.478; P = .17). The mean marbles fully covered did not differ significantly between groups (control: $t_{(10)}$ = .158; P = .877).

2.2. Neurobiological alterations: MS significantly reduces *Morc1*-mRNA expression in dams PFC' but not the hippocampal *Nr3c1* expression

Dams exposed to chronic separation from pups for four hours daily demonstrated significantly higher *Morc1* delta CT values compared to controls: $F_{(1, 15.5)} = 8.19$; $P < .01$ measured in the mPFC region (**Fig. 2A**).

No significant correlations were found with *Morc1* mRNA levels for the parameters with significant behavioral results (*Morc1* compared to LG: r= -.04; Arched-back nursing: r=.34; PD20 22-kHz: r=.14; PD20 50-kHz r= .02). Interestingly, *Morc1* levels showed significant correlations with the parameters of self-grooming, first pup retrieval and complete pup retrieval at the last day measured (*Morc1* and Self-grooming at PD20: r= -.594, P = 0.04; *Morc1* and first pup retrieval at PD20: r= -.814, P = 0.01; *Morc1* and complete pup retrieval at PD20: r= -.922, P = 0.01). However, after correcting for multiple testing these values did not reach significance. Furthermore, no significant correlations with *Morc1* mRNA levels were detected (*Morc1* to marbles fully buried: r=-0.33 and EPM time spent in open arms: r=-0.127).

However, we could not detect significant alterations in the hippocampus *Nr3c1* mRNA expression between groups ($F_{(1, 14)} = .00$; $P = .99$) (**Fig. 2B**).

2.3. Alterations in serum GABA and glutamate levels in dams

The one-Way ANOVA analysis for elevations did only reach statistical significance for GABA ($F_{(1, 27)} = 4.29$; $P = 0.048$) but not for glutamate in ($F_{(1, 27)} = 0.14$; $P = 0.72$) (**Fig. 2C+D**).

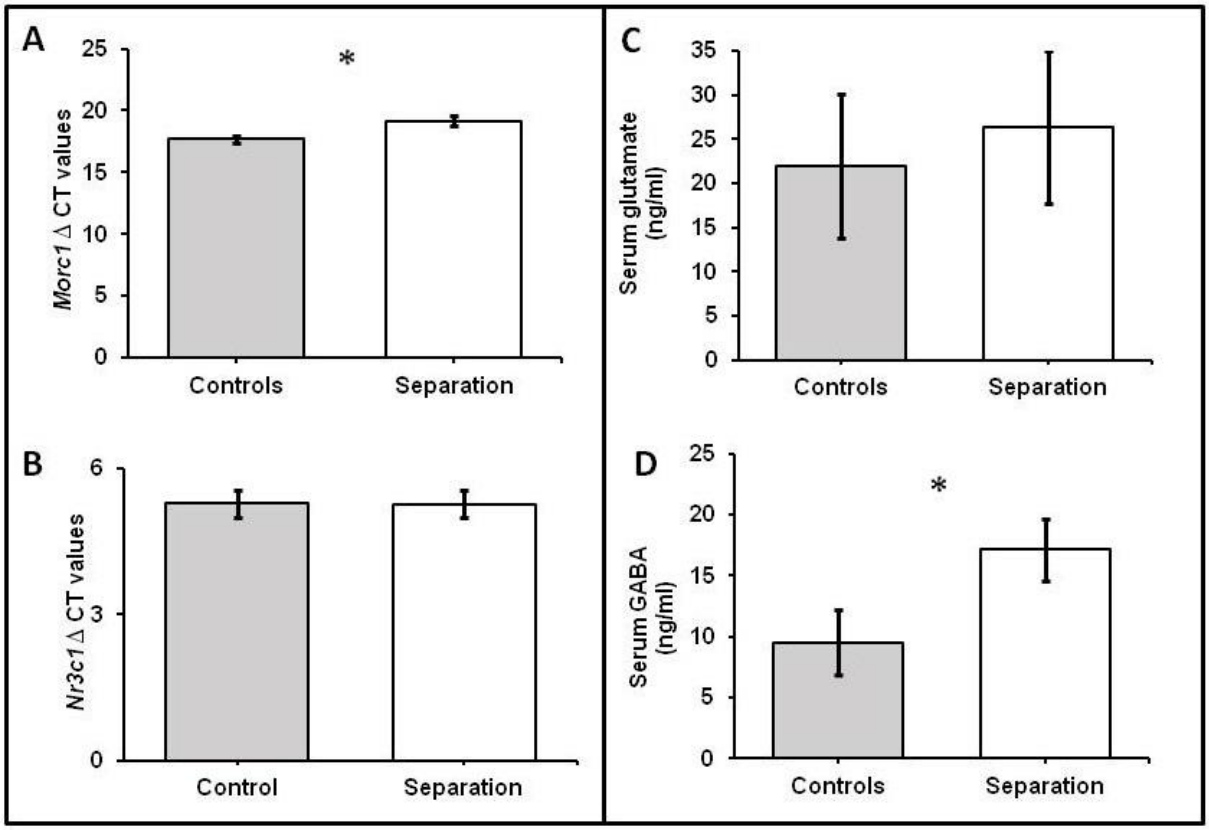


Fig. 2A: Delta CT values of *Morc1* mRNA analyzed in mPFC brain region. Separated dams were expressing significantly lower levels of *Morc1* mRNA as shown in significant higher CT values analyzed by quantitative real-time PCR. Means ± SEM are presented for n = 15 controls and n = 13 separated dams. Data was analyzed by One-Way ANOVA. *P < .01
2B: Delta CT values of *Nr3c1* mRNA analyzed in hippocampus brain region. Means ± SEM are presented for n = 8 controls and n = 8 separated dams. Data was analyzed by One-Way ANOVA.

Fig. 2C+D: Serum analyses of neurotransmitters glutamate for n = 14 controls and n = 15 separated dams (**C**) and GABA for n = 15 controls and n = 14 separated dams (**D**) after MS. Separated dams showed higher concentrations of both neurotransmitters but only GABA demonstrating significant differences. Means ± SEM are presented. Data was analyzed using a One-Way ANOVA.

3. Discussion

MS lead to overall increased maternal care and increased number of calls emitted after reunion. Testing for changes in anxiety behavior did not show significant differences between groups. However, anxiety behavior might be influenced by estrus as dams tested in the proestrus generally spent more time in the open [34]. Moreover, most studies using the long MS paradigm did not find any alterations in anxiety-like behavior in dams [35,36] suggesting no direct effect of MS on anxiety state of dams.

Neurobiological measurements revealed less *Morc1* mRNA in the mPFC after MS exposure but no differences in hippocampal GR mRNA expression. Regarding serum level

analysis, significantly higher GABA levels were found in dams subjected to MS compared to controls whereas glutamate levels did not differ.

It seems that MS in rat dams during the vulnerable time of postpartum can be seen analogous to the vulnerable timeframe of pups. The postpartum period is characterized by several physiological changes that make women prone to develop hormonal and neurochemical imbalances triggering psychiatric disorders and respective symptoms [37].

As highlighted in other studies, we demonstrated that separated rat dams showed altered behavior towards the pups after experiencing separation stress. In this study, the separated dams increased the maternal care towards their pups upon reunion whereas controls significantly demonstrated higher levels of self-maintenance. Thus, the chronic postpartum stress and subsequent induction of maternal care might compensate and buffer the stress of forced separation not only on maternal side, but also to buffer stress-related responses on pups with maternal care as a possible mediating factor. Of note, our MS protocol proceeds very similar to the protocol of other groups [4,7]. However, several differences in time and length of separations were detected [38]. Contrary to other groups [39], the dams were still maternal until the last day of testing.

Increased maternal care found in MS dams is in line with increased positive-related 50-kHz USV upon reunion. This might illustrate that the reunion with pups is more rewarding after a long time of absence. Surprisingly, the 22-kHz frequencies at PD20 were also higher in separated dams implying that separation has also led to negative mood-alterations compared to controls. Dams might be overstrained by their pups in a stress-induced mood, therefore increase the number of negatively associated affective calls as well.

On the other hand, the emitted USVs could also serve as communication tool towards the pups[40]. This could imply on an affective dimension that the separation procedure is not severely limited by adaptation of dams to separation protocol but rather induces more positive and negative-related USVs in long-term as detected in a non-invasive way of recording.

Of note, we have seen an increase in the number of 50-kHz frequencies over time which might imply the later tests were conducted with dams, the more positive emission of USVs were done by the dam upon reunion. Therefore, a constant positive affect after reunion with pups is noticeable, which is surprising giving the fact that with aging of pups, the tendency to show maternal care decreases over time. This might indicate a disruption on behavioral level. However, the reason for the increase of maternal care in the context of MS paradigm needs further elucidation. PD20 as the final day of testing showed significant results on both frequency ranges, but not at the first day of testing (PD2). This can show possible alterations in

affective state that is induced not immediately but rather through the repeated, chronic stress of separation. Kalinichev and colleagues also demonstrated that rat dams were more likely to emit USVs after experiencing the long MS protocol compared to controls [41] underlying our results that rat dams were also affected by separation on mood level. The here found increase in 50-kHz calls are also in line with other groups [18].

Gene expression downregulation of *Morc1* in separated dams compared to controls was found. Downregulation of *Morc1* either by knock-out variants [25] or hypermethylation of *Morc1* DNA [22,23] has been associated with depressive symptoms in other studies.

As there were no significant differences in anxiety-like behavior between groups but significant differences in *Morc1* expression it might be possible that reduced *Morc1* expression could serve as a subclinical marker indicating changes before pathological alterations manifest in behavior. This would be in line with our previous study investigating healthy young adults with subclinical depressive symptoms [23]. The chronically stressed dams did not directly demonstrate depressive-like behavior while increasing care towards pups. Correlating *Morc1* expression and maternal behavior revealed rather negative associations between *Morc1* and self-grooming, first pup retrieval and complete pup retrieval at the last day measured which did not reach significance after correcting for multiple testing. Again, this might reinforce the assumption of a subclinical pathological picture induced by MS and *Morc1* as a potential regulator. Thus, the possible role of *Morc1* in subclinical or clinical depressive-like symptoms needs further investigation. However, *Morc1* might still be suitable for detecting stress-induced neurobiological alterations.

In contrary to *Morc1*, our results on GR mRNA expression in the hippocampus to analyze possible acute stress reaction adaptations in the central nervous system did not reveal significant differences between groups. This has also been shown in another study with mice dams after MS [42]. Nevertheless, contradictory results with higher and lower GR mRNA expression have been reported in dams experiencing MS [12,43]. As GR expression is a possible acute stress marker, [44] and is highly influenced by the maternal care the mother expresses upon reunion with pups [45], it is likely that upregulation driven by environmental factors compensate neuronal adaptation of respective receptor expression. However, GR self-regulates itself and possibly increases 1 to develop depression [46,47].

Potential peripheral markers for depression that were analyzed in this study were GABA und glutamate serum levels. Studies with depressed women showed that measurements of plasma GABA levels correlated with aggressiveness levels [33]. Thus, finding significantly higher GABA levels after MS reinforces the pathological effects of MS on dams described above.

However, other studies demonstrate that the severity of depression as a stress-related disorder and its symptoms is associated with glutamate levels in the blood [48,49].

The fact that some behavioral and biological pathology markers were detected (as increased maternal care and USV together with increased GABA serum levels as well as decrease *Morc1* mRNA) but no difference in others was shown (such as GR mRNA as well as glutamate serum levels together with no altered anxiety-like behavior) might indicate that long term MS exposure induces subclinical depressive changes but by the time of measurement only alters some markers and not all at once.

In conclusion, analyzing maternally separated dams on behavioral and neurobiological level can provide important information on the vulnerable time of postpartum. Investigating the maternal side might also prove necessary when analyzing the consequences of MS on pups. As maternal behavior is significantly increased after MS, this alteration might have effects on stress coping of the pups. Analyzing USVs of dams upon reunion with pups proves to be an easy-to-apply and reliable tool to characterize affective states of dams. Combining behavioral and affective analyses can expand our knowledge on several stress-related disorders in animal models and humans.

Besides the sensitive neonatal period, is seems that the sensitive postpartum time is vulnerable to stress-induced alterations in *Morc1* expression and GABA serum levels as well. Further steps to characterize pathological effects of MS on dams should include more behavioral analysis on depressive-like behavior.

4. Materials and Methods

4.1. Animals

Experiments were conducted under the principles of Germany's Animal Welfare Act after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Northrhine-Westfalia). 32 timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Sulzfeld, Germany) arrived at gestational days 13 to 15. Pregnant dams were housed separately in plastic cages at controlled room temperatures (22°C) and humidity conditions (22 ± 2°C and 55 ± 25 %) with standard lighting (12h light and 12 hours of dark cycle, lights on at 11.00 pm). Dams were randomly assigned to MS or control group by a noninvolved colleague. The day of birth was considered the postnatal day (PD) 0. At PD2 pups were sexed and culled to 10 pups (if possible, pups were culled to five males and five female pups).

Animals were tested in two cohorts. Cohort 1 (N=16) did not undergo behavioral testing but were analyzed for brain and serum alterations. Cohort 2 (N=16) was subjected to behavioral

testing and brain and serum were analyzed. The sample size was chosen using G*Power statistical power analysis to ensure adequate power to detect a specific effect [50].

4.2. Maternal separation

MS dams (n=16) were separated from their pups for four hours daily from PD2 - 20 [47,51]. They were placed in a separate adjacent cage (with free access to food and water *ad libitum*) in the same room with the separated pups. After the separation, both dams and pups were returned to their home cages.

In controls (n=16), the dam and pups were only separated for weighing for a few minutes every 4th day. In the next step, another set of 16 animals (after detecting alterations in neurobiology and serum) was tested to correlate finding with behavior (n=8 controls and n=8 MS).

At PD21 pups were weaned and dams (n=16, Cohort 2) were behavioral tested. All dams were anesthetized with an intraperitoneal injection of ketamine and xylazine (ratio of 1:2) and sacrificed by decapitation for serum and brain extraction. Blood was centrifuged at room temperature at 1100 g for 20 minutes to extract Serum which was stored at −80°C. Dissected brains were stored at -80°C for later preparation of hippocampus and mPFC. Estrus cycle was determined according to the protocol of Marcondes and colleagues [52] to control for cycle state influences. All dams were proestrus.

4.3. Maternal behavior

Maternal behavior assessment was conducted during the dark phase. Animals could habituate to the experimental room for 20 - 30 minutes before testing. On PD 2, 6, 14 and 21 mother-pup interactions of separated and control dams were videotaped for 15 minutes after reunion with pups. Two high-resolution SONY cameras were focusing two sides of the maternal cage (one camera from the front and another from side in approximately 20 cm distance). Maternal behavior parameters included: active nursing (the dam actively arched-back over pups enabling access to dams' nipples [53], licking and grooming of pups (either head or anogenital region of pups), retrieval time of first pup and time retrieve complete litter. Furthermore, measuring time carrying pups from outside to inside the nest and time of regrouping within the nest was conducted. Parameters for self-maintenance comprised time dams spent self-grooming and rearing (with at least one paw off the ground). Maternal behavior was analyzed manually by the experimenter blinded to condition. In addition, all videos were analyzed by an independent party for subjective accuracy.

4.4. Ultrasonic vocalizations (USVs)

USVs have proven to reflect rodents' affective state in a non-invasive and accurate way [54]. While positive stimuli elicit shorter 50-kHz frequency ranges (33 – 90 kHz), negative and aversive stimuli are associated with 22-kHZ frequency spans (15 – 32.9 kHz)[19]. To assess relative changes, each dams' USVs were counted 15 minutes after reunion respectively. Real-time recording with Batlogger M (Elekon AG, Lucerne, Switzerland) was positioned rectangular above the cage in approximately 10 cm height on PD 2, 6, 14 and 21 (Fig. 2). The minimum frequency was set at 15 kHz and maximum frequency at 155 kHz with Volume = 2 and automatic setup trigger. Recorded USVs were analyzed using BatExplorer with FFT size of 1024 and overlap of 80% (Elekon AG, Lucerne, Switzerland). To distinguish between dams' and pups' USVs, non-frequency-modulated calls were regarded maternal whereas pups demonstrated atypical and U-shaped calls [55].

4.5. Anxiety-like behavioral measurements

To assess alterations in anxiety-like behavior after MS, elevated plus maze (EPM) [56] and marble burying (MB)[57] were performed on the day of weaning (PD21). The EPM consisted of a plus-shaped maze in approximately 50 cm height. Two open arms (50 cm × 10 cm) and two closed arms (50 cm × 10 cm) faced in opposite directions. In a five minutes trial time, the time spent in the open and closed arms were scored and the frequency of crossings within the open or close spaces was measured (dams started in the enclosed arms).

For the MB, 20 marbles were positioned symmetrically on a standard Macrolon IV cage on smoothened bedding. On the three fourth of the cage area, four rows of five marbles were positioned. After 15 minutes of trial times, the number of marbles buried by dams that were fully (100% invisible) or partially (more than 70% covered) was counted. The number of buried marbles serves as an indirect measure for anxiety-like behavior [57].

4.6. Real-time quantitative polymerase chain reaction (RT-PCR) of mPFC and hippocampus

To detect neurobiological alterations, mPFC and hippocampus were dissected using Paxinos and Watson's The Rat Brain in Stereotaxic Coordinates [58]. Isolation of RNA, DNA, and protein from hippocampus and mPFC samples were obtained using NucleoSpin® TriPrep (Macherey-Nagel, Düren, Germany) with slight modifications. For each sample, 40 µl of RNase-free water was added to obtain RNA. The concentration and quality of RNA was measured using 1 µl of the sample in NanoDrop™ ND-1000 Spectrophotometer (PEQLAB Biotechnologie, Erlangen,

Germany). For three mPFC samples, the amount of extracted mRNA was not sufficient for *Morc1* rtPCR analysis. Therefore, they were excluded.

To quantify mRNA levels, RNA was first reverse transcribed to cDNA using the High-Capacity RNA-to-cDNA™ Kit (Thermofisher Scientific, Darmstadt, Germany) according to the manufacturer's protocol. Then, 60 ng of cDNA to detect *Morc1* mRNA levels in the mPFC and 60ng of cDNA to detect *glucocorticoid-receptor* (*Nr3c1*) mRNA levels in the hippocampus were quantified. Only the animals from cohort 1 were examined for *Nr3c1* levels. The TaqMan hybridization with TaqMan™ Gene Expression Master Mix and primers using TaqMan gene expression assay for *Morc1* (Rn01474745_m1) and *Nr3c1* (Rn00561369_m1) were used. The number of target genes was normalized to the housekeeping genes *Glyceraldehyde-3-phosphate dehydrogenase* (Rn01775763_g1) and *Actin, beta* (Rn00667869_m1). The real-time PCR (Applied Biosystems 7500 Fast Real-Time PCR System) reaction was quantified by the number of cycle threshold (Delta CT method). All samples and standards were assayed in duplicates.

4.7. High-performance liquid chromatography (HPLC) for serum GABA and glutamate

HPLC grade acetonitrile (ACN) was obtained from VWR Chemicals (Langenfeld, Germany). Phtalaldehyd, 2-mercaptoethanol, glutamate, and GABA were ordered from SIGMA (Sigma-Aldrich, Taufkirchen, Germany). Furthermore, 1M sodium hydroxide (Waldeck, Münster, Germany), methanol (Carl Roth, Karlsruhe, Germany), perchloric acid (PCA) (Sigma-Aldrich, Steinheim, Germany), phosphor acid (J.T. Baker, Deventer, Netherlands) and 1M hydrochloric acid and sodium bicarbonate from stock were used. All reagents were of the highest purity available, and solely Milli-Q deionized water was used.

Dilution series for GABA and glutamate standards were created for every new mobile phase setup before samples were assessed. For analytic identification and quantification, GABA and glutamate in serum were identified by their characteristic retention times as determined by standard injections of GABA and glutamate. Calibration curve of standard solutions were obtained from a blank, and at least four standard solutions and the correlation coefficients of the curves were not less than 0.98. For sample preparation, 50 µl serum samples were deproteinized using an equal volume of 0.1M PCA to extract free amino acids. Serum sample and PCA were centrifuged (15 minutes, 10000 rpm, 4°C) and put on ice. Then, 30 µl of supernatant was loaded into the vials. For derivatization procedure, O-phtalaldehyde (OPA) was synthesized. Samples of each serum probe were conducted in duplicates, with two animal samples (one animal of each group) excluded due to technical errors and one sample per group for glutamate and GABA each. Therewith, serum of 14 control dams and 15 MS dams was

included for glutamate level analysis and serum of 15 control dams and 14 MS dams for GABA level analysis.

The serum glutamate and GABA protocol of Lee [59] was adapted with slight modifications: Two mobile phases with 5% (pH 3.54) and 28% ACN (pH 3.14) were set up separately using phosphoric acid to adjust pH (reverse phase chromatography).

Sample peak areas were measured via Labsolutions integrator system (LCsolution Version 1.24 SP1 Integration Time Program) and compared with the calibration curve standard to quantify respective concentrations. Data analysis parameters for area integration for all probes were determined with the width of 2000 seconds, the slope of 10 – 30 uV/min, the drift of 0 uV/min, the doubling time (T.DBL) of 1000 min, the minimum areas/height of 1000 counts. Moreover, the integration areas were calculated with horizontal baseline, and negative peaks were rejected.

HPLC analysis was performed with the HPLC system consisted of a LC10AB chromatograph, a RF-10A XL fluorescence detector, a LC-10 AD pump, a CBM-20A system controller, a CTO-20AC oven and a SIL-20AC autosampler system (Shimadzu, Langenfeld, Germany). All samples were injected and separated onto a reversed phase 5µ C18 (125 mm × 4 mm) column (Multospher® 100 RP 18-5µ) protected by a 30 µm pre-separation filter (Miltenyi Biotec, Germany).

4.8. Statistical analysis

For maternal care, a second independent rater reexamined all video material. Intraclass correlation (ICC) is an established statistical method to assess interrater differences of video material in subjective mother-infant relationships [60]. Only parameters showing an excellent inter-rater agreement [61] with r = >.75 were used for further analysis. Thus, the parameter carrying of pups (r=.59 and Cronbach's Alpha= .61) was excluded.

Each maternal care parameter was analyzed separately. For the analysis , 2 treatments (separation or control) × 4 time points (PD2, PD6, PD14, and PD20) were analyzed in a two-way, repeated measures Analysis of Variance for all parameters [35] and significance was set at $p < .00625$ to control for False Discovery Rate (FDR) when analyzing multiple comparisons (P-value < .05 divided by 2 treatments with 4 time points). Significant behavioral results were further assessed using t-test [62]. Analogous to maternal care videos, USVs were analyzed separately for the two frequency ranges using the repeated measures Analysis of Variance applying FDR, followed by student's t-test. Anxiety behavior was also assessed using student's t-test. Data was correlated using Pearson's correlation coefficient.

Brain and serum data were analyzed using a One-Way Analysis of Variance (ANOVA) with group as an independent variable (separation or control). GABA and glutamate serum concentrations, delta CT values of *Morc1* and *Nr3c1* as dependent variables were assessed using SPSS (IBM SPSS Statistics 25). Significance levels were set at p-value < .05

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

N.F., A.M. and I.B. designed the study. I.B. performed the experiments and was supported by A.M. The manuscript was written by I.B, A.M. and N.F. All authors approved the manuscript. This manuscript is our original work and it is submitted for first publication.

Abbreviations

ELS	early life stress
EPM	Elevated-plus maze test
FDR	False Discovery Rate
GABA	gamma-Aminobutyric acid
GR	Glucocorticoid-receptor
MB	Marble-burying test
MDD	Major Depressive Disorders
Morc1	MORC Family CW-Type Zinc Finger 1
Nr3c1	Nuclear Receptor Subfamily 3 Group C Member 1
MS	Maternal Separation
PD	Postnatal Day
PPD	Postpartum Depression
USV	Ultrasonic Vocalizations

References

1. Gareau, M. G., Jury, J., MacQueen, G., Sherman, P. M. & Perdue, M. H. Probiotic treatment of rat pups normalises corticosterone release and ameliorates colonic dysfunction induced by maternal separation. *Gut* **56**, 1522–1528 (2007).
2. Uhelski, M. L. & Fuchs, P. N. Maternal separation stress leads to enhanced emotional responses to noxious stimuli in adult rats. *Behav. Brain Res.* **212**, 208–212 (2010).
3. Veenema, A. H., Bredewold, R. & Neumann, I. D. Opposite effects of maternal separation on intermale and maternal aggression in C57BL/6 mice: link to hypothalamic vasopressin and oxytocin immunoreactivity. *Psychoneuroendocrinology* **32**, 437–450 (2007).
4. Freund, N., Thompson, B. S., DeNormandie, J., Vaccarro, K. & Andersen, S. L. Windows of vulnerability: Maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. *Neuroscience* (2013) doi:10.1016/j.neuroscience.2013.06.066.
5. Hock, E., McBride, S. & Gnezda, M. T. Maternal separation anxiety: Mother-infant separation from the maternal perspective. *Child Dev.* 793–802 (1989).
6. Aisa, B., Tordera, R., Lasheras, B., Del Río, J. & Ramírez, M. J. Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology* **32**, 256–266 (2007).

7. Lehmann, J. & Feldon, J. Long-term biobehavioral effects of maternal separation in the rat: Consistent or confusing? *Rev. Neurosci.* **11**, 383–408 (2000).
8. Lippmann, M., Bress, A., Nemeroff, C. B., Plotsky, P. M. & Monteggia, L. M. Long-term behavioural and molecular alterations associated with maternal separation in rats. *Eur. J. Neurosci.* (2007) doi:10.1111/j.1460-9568.2007.05522.x.
9. Monroy, E., Hernández-Torres, E. & Flores, G. Maternal separation disrupts dendritic morphology of neurons in prefrontal cortex, hippocampus, and nucleus accumbens in male rat offspring. *J. Chem. Neuroanat.* **40**, 93–101 (2010).
10. Brummelte, S. & Galea, L. A. M. Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. *Horm. Behav.* **58**, 769–779 (2010).
11. Macrì, S., Mason, G. J. & Würbel, H. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. *Eur. J. Neurosci.* **20**, 1017–1024 (2004).
12. Maniam, J. & Morris, M. J. Long-term postpartum anxiety and depression-like behavior in mother rats subjected to maternal separation are ameliorated by palatable high fat diet. *Behav. Brain Res.* (2010) doi:10.1016/j.bbr.2009.11.005.
13. Smith, J. W., Seckl, J., Evans, A. T., Costall, B. & Smythe, J. W. Gestational stress induces post-partum depression-like behaviour and alters maternal care in rats. *Psychoneuroendocrinology* **29**, 227–244 (2004).
14. Alves, R. L., Portugal, C. C., Summavielle, T., Barbosa, F. & Magalhães, A. Maternal separation effects on mother rodents' behaviour: A systematic review. *Neurosci. Biobehav. Rev.* (2019) doi:10.1016/j.neubiorev.2019.09.008.
15. Bousalham, R., Benazzouz, B., Hessni, A. E., Ouichou, A. & Mesfioui, A. Maternal Separation Affects Mothers ' Affective and Reproductive Behaviors as Well as Second Offspring ' s Emotionality. *Test* **2013**, 409–414 (2013).
16. Sung, Y. H. *et al.* Depression-like state in maternal rats induced by repeated separation of pups is accompanied by a decrease of cell proliferation and an increase of apoptosis in the hippocampus. *Neurosci. Lett.* **470**, 86–90 (2010).
17. Boccia, M. L. *et al.* Repeated long separations from pups produce depression-like behavior in rat mothers were analyzed. 65–71 (2007) doi:10.1016/j.psyneuen.2006.10.004.
18. Stevenson, C. W. *et al.* Neonatal maternal separation alters reward-related ultrasonic vocalizations in rat dams. *Behav. Brain Res.* **200**, 232–236 (2009).
19. Brudzynski, S. M. Pharmacology of Ultrasonic Vocalizations in adult Rats: Significance, Call Classification and Neural Substrate. *Curr. Neuropharmacol.* **13**, 180–192 (2015).
20. Heun-Johnson, H. & Levitt, P. Early-Life Stress Paradigm Transiently Alters Maternal Behavior, Dam-Pup Interactions, and Offspring Vocalizations in Mice. *Front. Behav. Neurosci.* **10**, 1–18 (2016).
21. Nagy, G., Banky, Z. & Takacs, L. Rapid decrease of plasma prolactin levels in lactating rats separated from their pups. *Endocrinol. Exp.* (1983).
22. Nieratschker, V. *et al. MORC1* exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. *Transl. Psychiatry* **4**, e429 (2014).
23. Mundorf, A., Schmitz, J., Güntürkün, O., Freund, N. & Ocklenburg, S. Methylation of MORC1: A possible biomarker for depression? *J. Psychiatr. Res.* **103**, 208–211 (2018).
24. Thomas, M. *et al.* Investigation of MORC1 DNA methylation as biomarker of early life stress and depressive symptoms. *J. Psychiatr. Res.* **120**, 154–162 (2020).
25. Schmidt, M. *et al.* Morc1 knockout evokes a depression-like phenotype in mice. *Behav. Brain Res.* **296**, 7–14 (2016).
26. Fogaça, M. V. & Duman, R. S. Cortical GABAergic dysfunction in stress and depression: new insights for therapeutic interventions. *Front. Cell. Neurosci.* **13**, 87 (2019).

27. Zhao, J. *et al.* Prefrontal alterations in GABAergic and glutamatergic gene expression in relation to depression and suicide. *J. Psychiatr. Res.* **102**, 261–274 (2018).
28. Kendell, S. F., Krystal, J. H. & Sanacora, G. GABA and glutamate systems as therapeutic targets in depression and mood disorders. *Expert Opin. Ther. Targets* **9**, 153–168 (2005).
29. Krystal, J. H. *et al.* Glutamate and GABA systems as targets for novel antidepressant and mood-stabilizing treatments. *Mol. Psychiatry* **7**, S71 (2002).
30. Hsu, F.-C. *et al.* Repeated neonatal handling with maternal separation permanently alters hippocampal GABAA receptors and behavioral stress responses. *Proc. Natl. Acad. Sci.* **100**, 12213–12218 (2003).
31. Odeon, M. M., Salatino, A. E. & Acosta, G. B. Consequences of postnatal stress: maternal separation in rats induces long-lasting changes on glutamate transporters. (2013).
32. Kim, S. Oxytocin and Postpartum Depression: Delivering on What's Known and What's Not. **64**, 2391–2404 (2015).
33. Bjork, J. M. *et al.* Plasma GABA levels correlate with aggressiveness in relatives of patients with unipolar depressive disorder. *Psychiatry Res.* **101**, 131–136 (2001).
34. Marcondes, F. K., Miguel, K. J., Melo, L. L. & Spadari-Bratfisch, R. C. Estrous cycle influences the response of female rats in the elevated plus-maze test. *Physiol. Behav.* **74**, 435–440 (2001).
35. Boccia, M. L. & Pedersen, C. A. Brief vs. long maternal separations in infancy: Contrasting relationships with adult maternal behavior and lactation levels of aggression and anxiety. *Psychoneuroendocrinology* **26**, 657–672 (2001).
36. Aguggia, J. P., Suárez, M. M. & Rivarola, M. A. Early maternal separation: neurobehavioral consequences in mother rats. *Behav. Brain Res.* **248**, 25–31 (2013).
37. Beck, C. T. Predictors of postpartum depression: An update. *Nurs. Res.* (2001) doi:10.1097/00006199-200109000-00004.
38. Tan, S., Ho, H. S., Song, A. Y., Low, J. & Je, H. S. Maternal Separation Does Not Produce a Significant Behavioral Change in Mice. *Exp. Neurobiol.* (2017) doi:10.5607/en.2017.26.6.390.
39. Champagne, F. A., Francis, D. D., Mar, A. & Meaney, M. J. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. *Physiol. Behav.* **79**, 359–371 (2003).
40. Yee, N., Schwarting, R. K., Fuchs, E. & Wöhr, M. Increased affective ultrasonic communication during fear learning in adult male rats exposed to maternal immune activation. *J. Psychiatr. Res.* **46**, 1199–1205 (2012).
41. Kalinichev, M., Easterling, K. W. & Holtzman, S. G. Periodic postpartum separation from the offspring results in long-lasting changes in anxiety-related behaviors and sensitivity to morphine in Long-Evans mother rats. *Psychopharmacology (Berl.)* **152**, 431–439 (2000).
42. Wei, L., David, A., Duman, R. S., Anisman, H. & Kaffman, A. Early life stress increases anxiety-like behavior in Balbc mice despite a compensatory increase in levels of postnatal maternal care. *Horm. Behav.* **57**, 396–404 (2010).
43. Orso, R. *et al.* Maternal behavior of the mouse dam toward pups: implications for maternal separation model of early life stress. *Stress* (2018) doi:10.1080/10253890.2017.1389883.
44. Liu, D. *et al.* Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science* **277**, 1659–1662 (1997).
45. Francis, D., Diorio, J., Liu, D. & Meaney, M. J. Nongenomic transmission across generations of maternal behavior and stress responses in the rat. *Science* **286**, 1155–1158 (1999).
46. Wang, H.-T., Han, F. & Shi, Y.-X. Activity of the 5-HT1A receptor is involved in the alteration of glucocorticoid receptor in hippocampus and corticotropin-releasing factor in hypothalamus in SPS rats. *Int. J. Mol. Med.* **24**, 227–231 (2009).

47. Zhang, T. Y., Labonté, B., Wen, X. L., Turecki, G. & Meaney, M. J. Epigenetic mechanisms for the early environmental regulation of hippocampal glucocorticoid receptor gene expression in rodents and humans. *Neuropsychopharmacology* **38**, 111 (2013).
48. Altamura, C. A., Mauri, M. C., Ferrara, A. & Moro, A. R. Plasma and platelet excitatory amino acids in psychiatric disorders. *Am. J. Psychiatry* **150**, 1731 (1993).
49. Mitani, H. *et al.* Correlation between plasma levels of glutamate, alanine and serine with severity of depression. *Prog. Neuropsychopharmacol. Biol. Psychiatry* **30**, 1155–1158 (2006).
50. Faul, F., Erdfelder, E., Lang, A.-G. & Buchner, A. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. *Behav. Res. Methods* **39**, 175–191 (2007).
51. Kalinichev, M., Easterling, K. W. & Holtzman, S. G. Periodic postpartum separation from the offspring results in long-lasting changes in anxiety-related behaviors and sensitivity to morphine in Long-Evans mother rats. *Psychopharmacology (Berl.)* **152**, 431–439 (2000).
52. Marcondes, F. K., Bianchi, F. J. & Tanno, A. P. Determination of the estrous cycle phases of rats: some helpful considerations. *Braz. J. Biol. Rev. Brasleira Biol.* **62**, 609–614 (2002).
53. Macrì, S., Mason, G. J. & Würbel, H. Dissociation in the effects of neonatal maternal separations on maternal care and the offspring's HPA and fear responses in rats. *Eur. J. Neurosci.* **20**, 1017–1024 (2004).
54. Simola, N. Rat Ultrasonic Vocalizations and Behavioral Neuropharmacology: From the Screening of Drugs to the Study of Disease. *Curr. Neuropharmacol.* (2015) doi:10.2174/1570159X13999150318113800.
55. Stevenson, C. W. *et al.* Neonatal maternal separation alters reward-related ultrasonic vocalizations in rat dams. *Behav. Brain Res.* **200**, 232–236 (2009).
56. Pellow, S., Chopin, P., File, S. E. & Briley, M. Validation of open : closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J. Neurosci. Methods* (1985) doi:10.1016/0165-0270(85)90031-7.
57. Malkova, N. V., Collin, Z. Y., Hsiao, E. Y., Moore, M. J. & Patterson, P. H. Maternal immune activation yields offspring displaying mouse versions of the three core symptoms of autism. *Brain. Behav. Immun.* **26**, 607–616 (2012).
58. Paxinos, G. & Watson, C. *The Rat Brain in Stereotaxic Coordinates*. (Elsevier, 2006).
59. Lee, H., Chang, M. J. & Kim, S. H. Effects of poly-γ-glutamic acid on serum and brain concentrations of glutamate and GABA in diet-induced obese rats. *Nutr. Res. Pract.* **4**, 23–29 (2010).
60. Hirschmann, N., Kastner-Koller, U., Deimann, P., Schmelzer, M. & Pietschnig, J. Reliable and valid coding of thin slices of video footage: Applicability to the assessment of mother-child interactions. *J. Psychopathol. Behav. Assess.* **40**, 249–258 (2018).
61. Cicchetti, D. V. Guidelines, criteria, and rules of thumb for evaluating normed and standardized assessment instruments in psychology. *Psychol. Assess.* **6**, 284 (1994).
62. Maniam, J. & Morris, M. J. Long-term postpartum anxiety and depression-like behavior in mother rats subjected to maternal separation are ameliorated by palatable high fat diet. *Behav. Brain Res.* (2010) doi:10.1016/j.bbr.2009.11.005.

Chapter 5 |
Biomarkers for psychiatric disorders

Methylation of Morc1: A possible biomarker for depression?

Annakarina Mundorf [a]*, Judith Schmitz [b]*, Onur Güntürkün [b], Nadja Freund [a] and Sebastian Ocklenburg [b]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany
[b] Institute of Cognitive Neuroscience, Department Biopsychology, Ruhr University Bochum, Germany

*joint first

Mundorf A, Schmitz J, Güntürkün O, Freund N, Ocklenburg S (2018). Methylation of MORC1: A possible biomarker for depression? *J Psychiatr Res*. Aug;103:208-211, https://doi.org/10.1016/j.jpsychires.2018.05.026

Journal of Psychiatric Research 103 (2018) 208–211

Contents lists available at ScienceDirect

Journal of Psychiatric Research

journal homepage: www.elsevier.com/locate/jpsychires

Methylation of *MORC1*: A possible biomarker for depression?

Annakarina Mundorf[a,1], Judith Schmitz[b,1], Onur Güntürkün[b], Nadja Freund[a,*,2], Sebastian Ocklenburg[b,2]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Universitätsstraße 150, 44780 Bochum, Germany
[b] Institute of Cognitive Neuroscience, Department Biopsychology, Ruhr University Bochum, Universitätsstraße 150, 44780 Bochum, Germany

ARTICLE INFO

Keywords:
Biomarker
Epigenetics
Beck depression inventory

ABSTRACT

New findings identified the *MORC1* gene as a link between early life stress and major depression. In this study, *MORC1* methylation was investigated in 60 healthy human adults (30 women, 30 men) between 19 and 33 years of age. For analysis, DNA was isolated from buccal cells. The results show that DNA methylation in the *MORC1* promoter region significantly correlates with the Beck Depression Inventory score in the examined non-clinical population. Sum score of birth complications, however, seems to correlate negatively with methylation. These findings further confirm that *MORC1* is a stress sensitive gene and a possible biomarker for depression.

1. Introduction

Major depressive disorder (MDD) is one of the leading causes of disability worldwide as 6–18% of the population will develop MDD during their life span (Drevets and Furey, 2009; World Health Organization, 2016). It has been widely shown that early life stress (ELS) contributes to the risk of developing MDD by inducing structural and functional changes in the brain (Carr et al., 2013; Kendler et al., 2004; Teicher et al., 2003). Recent data point to an involvement of the epigenome in explaining the link between ELS and MDD (Essex et al., 2013). Epigenetic changes after ELS could not only predispose for MDD, they might also have the potential to be used as early detection markers. Nieratschker and colleagues (Nieratschker et al., 2014) identified a relationship between ELS and an altered methylation state of the *MORC family CW-type zinc finger 1* (*MORC1*) gene (Nieratschker et al., 2014). In a cross-species, multi-tissue approach, the researchers investigated the effects of early life stress on DNA methylation. They compared DNA methylation from human cord blood following prenatal stress, the prefrontal cortex tissue of adult rats that had been exposed to prenatal stress, and blood cells of adolescent nonhuman primates after maternal separation with matched non-stressed control groups. Significantly reduced methylation of the *MORC1* gene was found in all tissues of all species (Nieratschker et al., 2014). To further investigate these results, the authors performed a gene-based case-control analysis utilizing data from a previous GWAS MDD study. Specific gene variants of the *MORC1* gene were associated with MDD. The link between *MORC1* and MDD was supported by a study by Schmidt et al., in 2016. In female MORC1 knockout mice, depressive-like behavior was observed, as well as differences in *BDNF* mRNA levels in the hippocampus (Schmidt et al., 2016). Of interest, differential *BDNF* expression has also been previously reported in MDD patients (Jiang and Salton, 2013).

The *MORC* gene was first discovered in 1998 by Watson and colleagues. It was attributed a major role in spermatogenesis based on findings that male *MORC*−/− mice were infertile while female *MORC*−/− mice did not show any symptoms (Watson et al., 1998). The *MORC* gene family exists only in meiotic and mitotic germ cells, further suggesting importance in male mouse spermatogenesis. However, given that other closely related genes of human *MORC*, such as human *KIAA0852* and *KIAA0136,* are found in several somatic tissues, it seemed likely that *MORC* also exists in somatic tissues (Inoue et al., 1999). In recent years, the *MORC* family has been characterized more precisely, with seven defined members; *MORC1, MORC2, MORC3, MORC4, MORC5, MORC6* and *MORC7* (Li et al., 2013; Moissiard et al., 2014). Moreover, the role of *MORCs* was specialized to epigenetic regulation in diverse nuclear processes due to the special domain architecture of this highly conserved nuclear protein superfamily (Li et al., 2013). This protein domain also suggests a connection with either chromatin methylation status or early embryonic development, reinforcing its reported association with ELS (Perry and Zhao, 2003).

Some members of the *MORC* family have already been characterized more precisely in plants and mammals, with implied roles in transcription repression, e.g. *MORC2* in human cancer cells, and gene

* Corresponding author.
E-mail address: nadja.freund@ruhr-uni-bochum.de (N. Freund).
[1] Joint first author.
[2] Joint senior author.

https://doi.org/10.1016/j.jpsychires.2018.05.026
Received 8 January 2018; Received in revised form 28 May 2018; Accepted 29 May 2018

silencing (Brabbs et al., 2013; Liggins et al., 2007; Quinlan et al., 2006; Wang et al., 2010). Given its potential role in inferring susceptibility for MDD after ELS (Nieratschker et al., 2014; Schmidt et al., 2016) we confirmed the expression of MORC1 protein in the brain and its presence during embryonic development (shown in the rodent brain, unpublished data).

In the present study we analyzed *MORC1* gene methylation in buccal cell samples from a non-clinical population. Methylation status was correlated with the scores in the Beck Depression Inventory to investigate whether *MORC1* methylation is associated with a subclinical depressive phenotype. We also examined whether *MORC1* methylation is correlated with complications during birth as one factor of ELS. This research has the potential to deepen our understanding regarding the association between ELS and the risk to develop MDD, and the potential of *MORC1* to act as a marker for predicting this risk.

2. Material and methods

We investigated *MORC1* methylation in 60 healthy human adults (30 women, 30 men) between 19 and 33 years of age (mean = 24.40, SD = 3.08). All participants gave written informed consent. The local ethics committee of the Psychological Faculty at Ruhr University Bochum approved the study procedure. Birth complications were assessed via questionnaires completed by participants and their parents. Reported items included preterm birth (before completion of the 37th week of gestation), long labour, multiple birth, caesarian birth, forceps used, breech presentation, Rhesus incompatibility, and others according to Bailey and McKeever (2004). A sum score was calculated for each participant (mean 0.52, SD = 0.89). Participants also completed the Beck Depression Inventory (BDI) (Beck et al., 1996). DNA was isolated from buccal cells using the blackPREP Swab DNA Kit (Analytik Jena, Germany), bisulfite converted with the EpiTect Kit (Qiagen, Germany) and analyzed using the Illumina MethylationEPIC array (Illumina, United States). Quality control, preprocessing and processing were performed using RStudio version 0.99.903 (RStudio, Inc., United States) and RnBeads (Max Planck Institute for Informatics, Germany) (Assenov et al., 2014). The promoter region of the *MORC1* gene was defined as 1.5 kb upstream and 0.5 kb downstream of the transcription start site. The array covered 13 individual CGsites within this region (chr3: 108836490–108838489). Statistical analysis was conducted with the whole dataset and after elimination of all data points smaller than three SD below or larger than 3 SD above the mean DNA methylation for each CGsite. In order to control for possible sex differences, we conducted t-tests for independent samples between the sexes regarding the sum of birth complications, BDI scores, DNA methylation levels at the 13 CGsites and mean *MORC1* promoter region methylation. To control for age effects, we correlated age with DNA methylation in the CGsites and the whole promoter. We used Pearson correlation to investigate the relationship between birth complication sum score and DNA methylation levels at the 13 CGsites using Bonferroni correction for multiple comparisons ($\alpha = 0.0038$) and between birth complication sum score and mean *MORC1* promoter region methylation. Furthermore, we conducted a step-wise linear regression analysis with DNA methylation levels in all 13 CGsites as predictors and BDI score as the dependent variable. Pearson correlation was also used to investigate the relationship between birth complication sum score and BDI score.

3. Results

We did not find sex differences in birth complication sum score, BDI scores, or mean *MORC1* promoter region methylation (all $p > .05$). Only CGsite cg07090057 showed a statistically significant sex difference (males: mean = 0.926, SD = 0.016; females: mean = 0.937, SD = 0.012; $t(58) = 3.22$, $p < .01$), while the remaining 12 CGsites did not (all $t(58) < 1.4$, all $p > .05$). These results did not change after the elimination of outliers. CGsite cg04167867 showed a nominally significant correlation with age (r = -.280, $p < .05$), but no CGsite reached significance after controlling for multiple comparisons. After elimination of outliers, no CGsite significantly correlated with age.

Birth complication sum scores were correlated with DNA methyla-tion levels in 13 individual CGsites within the *MORC1* promoter region (chr3: 108836490–108838489) using Bonferroni correction for mul-tiple comparisons ($\alpha = 0.0038$). We also correlated birth complication scores with the mean value of DNA methylation for the whole promoter region. We found a significant negative correlation between birth complication sum score and *MORC1* promoter region total methylation (r = -.276, $p = .033$) as well as *MORC1* CGsite cg18733433 methyla-tion (r = -.373, $p = .003$). Regression analysis with DNA methylation levels from all 13 CGsites as predictors and BDI score as the dependent variable reached significance ($F(1,58) = 4.63$, $p = .036$) with R = 0.27 and $R^2 = 0.07$. The CGsite cg27175191 was significantly positively associated with BDI score individually ($\beta = 0.27$, $t = 2.15$, $p = .036$).

After elimination of outliers, the correlation between birth compli-cation sum score and *MORC1* promoter region methylation (r = -.101, $p = .443$) and *MORC1* CGsite cg18733433 methylation (r = -.083, $p = .531$) did not remain significant. However, the association between DNA methylation and BDI score remained significant ($F(1,51) = 4.69$, $p = .035$) with R = 0.29 and $R^2 = 0.08$. Again, CGsite cg27175191 reached individual significance ($\beta = 0.29$, $t = 2.17$, $p = .035$; see Fig. 1). Birth complication sum scores were not correlated with BDI score (r = −0.37, $p = .780$).

4. Discussion

The present data show that DNA methylation in the *MORC1* pro-moter region is significantly associated with BDI scores in a non-clinical population (Fig. 1). The BDI scores in our sample ranged between 0 and 15, which was expected with regard to the sample composition. Scores below 10 are considered as revealing no or minimal depression, while scores between 10 and 18 are considered as indicating mild to moderate depression (Beck et al., 1988). While our findings do not allow us to draw conclusions regarding the pathological mechanisms of *MORC1*, they might be indicative of an epigenetic regulation of *MORC1* by subtle environmental influences that induce sensitivity for subclinical depressive symptoms, possibly in line with a predisposition for MDD. This data set extends the known involvement of the *MORC1* gene in MDD patients (Nieratschker et al., 2014) to subclinical symptoms in

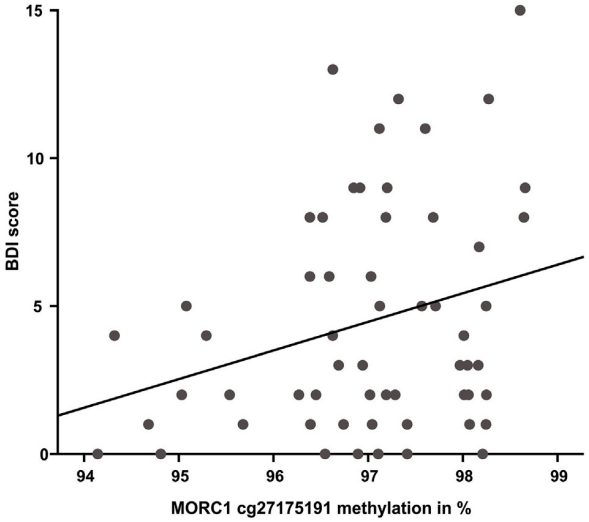


Fig. 1. Relationship between *MORC1* cg27175191 methylation in % and BDI score after elimination of outliers. The β value reached significance ($\beta = 0.29$, $p = .035$).

non-clinical subjects.

Higher methylation is traditionally associated with lower protein expression (Razin and Riggs, 1980) and therefore we can speculate that lower amounts of *MORC1* expression may infer higher levels of depressive symptoms (e.g. BDI scores in our sample). This is in line with the fact that mice lacking the MORC1 protein show a depressive-like phenotype (Schmidt et al., 2016).

In contrast, previously reported hypomethylation following ELS (Nieratschker et al., 2014), would suggest that decreased methylation of *MORC1* is associated with depression as ELS is a strong predictor of MDD (Carr et al., 2013; Leussis et al., 2012; Putnam, 2003). Interestingly, our data set shows that a sum score of birth complications is negatively correlated with DNA methylation of *MORC1* in buccal cells of healthy human adults. This finding applies to the overall *MORC1* promoter methylation as well as to CG site cg18733433 and is in line with hypomethylation after ELS (Nieratschker et al., 2014) when we consider birth complications as ELS. However, the effect in our data did not remain significant after the elimination of outliers, which makes it likely that it was partly driven by extreme values. Nevertheless, it is still an interesting result in line with the literature that requires replication in larger samples.

In general, reduced DNA methylation in *MORC1* following ELS contrasts the correlation we report between *MORC1* hypermethylation and BDI, leaving the role of *MORC1* in connecting ELS and MDD unclear. Our data set did not show a correlation between ELS (defined by birth complications) and BDI scores. Therefore, different mechanisms might be responsible for variation in methylation following ELS and methylation correlated with BDI. We also did not find a significant moderation effect for each CGsite with DNA methylation. This lack of significance may be due to different CGsites within the *MORC1* promoter region being associated with birth complication sum score and BDI scores. A further limitation which may explain the lack of correlation between BDI scores and birth complications is the self-reported measurement of birth complications and BDI by the participants.

Birth complications are just one specific adverse early life event, whilst many have been reported, particularly in the context of mental health outcomes. Furthermore, we did not control for factors which may confer a predisposition to birth complications, or any other risk exposures during pregnancy, such as maternal depression, smoking, and maternal behavior after birth. This is important, as it is known that maternal stress and prenatal stress, as well as MDD of the mother, is associated with differential DNA methylation of the offspring (Abbott et al., 2018; Bodnar et al., 2009; Vidal et al., 2014). Therefore, the correlation between the birth complications and *MORC1* methylation might not be the result of ELS but can potentially be explained by parental behavior or genetic factors that correlate with birth complications. However, in the study of Nieratschker et al. (2014), ELS was well defined in the animal studies and the hypomethylation after ELS was found in three different cell types and species, indicating strong and well-preserved mechanisms.

As DNA methylation is tissue-specific, the use of buccal cells for the investigation of birth complications and BDI may not be representative of DNA methylation signatures in our tissue of interest (the brain). However, a recent study showed that DNA methylation in peripheral tissue can reveal information on CNS-related phenotypes but should rather be interpreted as epigenetic signatures (Freytag et al., 2017). Furthermore, despite the limitations for our interpretations of our results regarding birth complications, we could extend the known connection between ELS and *MORC1* methylation that was found in $CD34^+$ cells, CD3-T cells and brain tissue (Nieratschker et al., 2014) to buccal cells.

We report a significant correlation between *MORC1* hypermethylation and BDI, giving hope for a reliable biomarker for MDD. As our results were found in buccal cells, *MORC1* hypermethylation could be an easy to apply, noninvasive biomarker for detecting subclinical depressive phenotype and therefore help to predict the risk of developing MDD.

Longitudinal studies spanning a range of developmental ages, and studies with clinical samples might improve our understanding of the connection between *MORC1* methylation status and the risk to develop MDD. Moreover, they will be essential in confirming *MORC1* methy-lation as a predictor of MDD risk.

Funding

This work was partly supported by the Mercator Research Center Ruhr (Project number GZ: An-2015-0061).

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments:

We would like to thank Susanna Roberts for proofreading and helpful comments on the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jpsychires.2018.05.026.

References

Abbott, P.W., Gumusoglu, S.B., Bittle, J., Beversdorf, D.Q., Stevens, H.E., 2018. Prenatal stress and genetic risk: how prenatal stress interacts with genetics to alter risk for psychiatric illness. Psychoneuroendocrinology 90, 9–21. https://doi.org/10.1016/j.psyneuen.2018.01.019.

Assenov, Y., Müller, F., Lutsik, P., Walter, J., Lengauer, T., Bock, C., 2014. Comprehensive analysis of DNA methylation data with RnBeads. Nat. Methods 11, 1138–1140. https://doi.org/10.1038/nmeth.3115.

Bailey, L.M., McKeever, W.F., 2004. A large-scale study of handedness and pregnancy/birth risk events: implications for genetic theories of handedness. Laterality 9, 175–188. http://dx.doi.org/10.1080/13576500342000013.

Beck, A., Steer, R.A., Brown, G.K., 1996. Beck Depression Inventory, second ed. Manual. Psychol. Corp, San Antonio TX.

Beck, A.T., Steer, R.A., Carbin, M.G., 1988. Psychometric properties of the Beck depression inventory: twenty-five years of evaluation. Clin. Psychol. Rev. 8, 77–100. https://doi.org/10.1016/0272-7358(88)90050-5.

Bodnar, L.M., Wisner, K.L., Moses-Kolko, E., Sit, D.K.Y., Hanusa, B.H., 2009. Prepregnancy body mass index, gestational weight gain, and the likelihood of major depressive disorder during pregnancy. J. Clin. Psychiatr. 70, 1290–1296. https://doi.org/10.4088/JCP.08m04651.

Brabbs, T.R., He, Z., Hogg, K., Kamenski, A., Li, Y., Paszkiewicz, K.H., Moore, K.A., O'Toole, P., Graham, I.A., Jones, L., 2013. The stochastic silencing phenotype of Arabidopsis morc6 mutants reveals a role in efficient RNA-directed DNA methylation. Plant J. Cell Mol. Biol. 75, 836–846. https://doi.org/10.1111/tpj.12246.

Carr, C.P., Martins, C.M.S., Stingel, A.M., Lemgruber, V.B., Juruena, M.F., 2013. The role of early life stress in adult psychiatric disorders: a systematic review according to childhood trauma subtypes. J. Nerv. Ment. Dis. 201, 1007–1020. https://doi.org/10.1097/NMD.0000000000000049.

Drevets, W.C., Furey, M.L., 2009. Depression and the brain. In: Squire, L.R. (Ed.), Encyclopedia of Neuroscience. Academic Press, Oxford, pp. 459–470. https://doi.org/10.1016/B978-008045046-9.00846-9.

Essex, M.J., Boyce, W.T., Hertzman, C., Lam, L.L., Armstrong, J.M., Neumann, S.M.A., Kobor, M.S., 2013. Epigenetic vestiges of early developmental adversity: childhood stress exposure and DNA methylation in adolescence. Child Dev. 84, 58–75. https://doi.org/10.1111/j.1467-8624.2011.01641.x.

Freytag, V., Carrillo-Roa, T., Milnik, A., Sämann, P.G., Vukojevic, V., Coynel, D., Demougin, P., Egli, T., Gschwind, L., Jessen, F., Loos, E., Maier, W., Riedel-Heller, S.G., Scherer, M., Vogler, C., Wagner, M., Binder, E.B., de Quervain, D.J.-F., Papassotiropoulos, A., 2017. A peripheral epigenetic signature of immune system genes is linked to neocortical thickness and memory. Nat. Commun. 8 (15193). https://doi.org/10.1038/ncomms15193.

Inoue, N., Hess, K.D., Moreadith, R.W., Richardson, L.L., Handel, M.A., Watson, M., Zinn, A.R., 1999. New gene family defined by MORC, a nuclear protein required for mouse spermatogenesis. Hum. Mol. Genet. 8, 1201–1207. https://doi.org/10.1093/hmg/8.7.1201.

Jiang, C., Salton, S.R., 2013. The role of neurotrophins in major depressive disorder. Transl. Neurosci. 4, 46–58. https://doi.org/10.2478/s13380-013-0103-8.

Kendler, K.S., Kuhn, J.W., Prescott, C.A., 2004. Childhood sexual abuse, stressful life events and risk for major depression in women. Psychol. Med. null 1475–1482. https://doi.org/10.1017/S003329170400265X.

Leussis, M.P., Freund, N., Brenhouse, H.C., Thompson, B.S., Andersen, S.L., 2012. Depressive-like behavior in adolescents after maternal separation: sex differences, controllability, and GABA. Dev. Neurosci. 34, 210–217. https://doi.org/10.1159/000339162.

Li, D.-Q., Nair, S.S., Kumar, R., 2013. The MORC family: new epigenetic regulators of transcription and DNA damage response. Epigenetics 8, 685–693. https://doi.org/10.4161/epi.24976.

Liggins, A.P., Cooper, C.D.O., Lawrie, C.H., Brown, P.J., Collins, G.P., Hatton, C.S., Pulford, K., Banham, A.H., 2007. MORC4, a novel member of the MORC family, is highly expressed in a subset of diffuse large B-cell lymphomas. Br. J. Haematol. 138, 479–486. https://doi.org/10.1111/j.1365-2141.2007.06680.x.

Moissiard, G., Bischof, S., Husmann, D., Pastor, W.A., Hale, C.J., Yen, L., Stroud, H., Papikian, A., Vashisht, A.A., Wohlschlegel, J.A., Jacobsen, S.E., 2014. Transcriptional gene silencing by Arabidopsis microrchidia homologues involves the formation of heteromers. Proc. Natl. Acad. Sci. U.S.A. 111, 7474–7479. https://doi.org/10.1073/pnas.1406611111.

Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nöthen, M.M., Suomi, S.J., Peus, V., Scharnholz, B., Dukal, H., Hohmeyer, C., Wolf, I. a.-C., Cirulli, F., Gass, P., Sütterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Transl. Psychiatry 4, e429. https://doi.org/10.1038/tp.2014.75.

Perry, J., Zhao, Y., 2003. The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. Trends Biochem. Sci. 28, 576–580. https://doi.org/10.1016/j.tibs.2003.09.007.

Putnam, F.W., 2003. Ten-year research update review: child sexual abuse. J. Am. Acad. Child Adolesc. Psychiatry 42, 269–278. https://doi.org/10.1097/00004583-200303000-00006.

Quinlan, K.G.R., Verger, A., Kwok, A., Lee, S.H.Y., Perdomo, J., Nardini, M., Bolognesi, M., Crossley, M., 2006. Role of the C-terminal binding protein PXDLS motif binding cleft in protein interactions and transcriptional repression. Mol. Cell Biol. 26, 8202–8213. https://doi.org/10.1128/MCB.00445-06.

Razin, A., Riggs, A.D., 1980. DNA methylation and gene function. Science 210, 604–610.

Schmidt, M., Brandwein, C., Luoni, A., Sandrini, P., Calzoni, T., Deuschle, M., Cirulli, F., Riva, M.A., Gass, P., 2016. Morc1 knockout evokes a depression-like phenotype in mice. Behav. Brain Res. 296, 7–14. https://doi.org/10.1016/j.bbr.2015.08.005.

Teicher, M.H., Andersen, S.L., Polcari, A., Anderson, C.M., Navalta, C.P., Kim, D.M., 2003. The neurobiological consequences of early stress and childhood maltreatment. Neurosci. Biobehav. Rev. 27, 33–44.

Vidal, A.C., Benjamin Neelon, S.E., Liu, Y., Tuli, A.M., Fuemmeler, B.F., Hoyo, C., Murtha, A.P., Huang, Z., Schildkraut, J., Overcash, F., Kurtzberg, J., Jirtle, R.L., Iversen, E.S., Murphy, S.K., 2014. Maternal stress, preterm birth, and DNA methylation at imprint regulatory sequences in humans. Genet. Epigenet. 6, 37–44. https://doi.org/10.4137/GEG.S18067.

Wang, G.-L., Wang, C.-Y., Cai, X.-Z., Chen, W., Wang, X.-H., Li, F., 2010. Identification and expression analysis of a novel CW-type zinc finger protein MORC2 in cancer cells. Anat. Rec. Hoboken NJ 2007 (293), 1002–1009. https://doi.org/10.1002/ar.21119.

Watson, M.L., Zinn, A.R., Inoue, N., Hess, K.D., Cobb, J., Handel, M.A., Halaban, R., Duchene, C.C., Albright, G.M., Moreadith, R.W., 1998. Identification of morc (microrchidia), a mutation that results in arrest of spermatogenesis at an early meiotic stage in the mouse. Proc. Natl. Acad. Sci. U.S.A. 95, 14361–14366.

World Health Organization, 2016. WHO | Depression [WWW Document]. WHO. http://www.who.int/mediacentre/factsheets/fs369/en/, Accessed date: 29 August 2016.

MORC1 methylation and BDI are associated with microstructural features of the hippocampus and medial prefrontal cortex

Annakarina Mundorf [a*], Judith Schmitz [b], Karola Hünten [a], Christoph Fraenz [c], Caroline Schlüter [c], Erhan Genc [c], Sebastian Ocklenburg [c] and Nadja Freund [a]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany

[b] School of Medicine, University of St Andrews, United Kingdom

[c] Institute of Cognitive Neuroscience, Department Biopsychology, Ruhr University Bochum, Germany

*Corresponding author

Research article / in preparation

Abstract

Background: Alterations in the hippocampus and prefrontal cortex (PFC) have frequently been reported in depressed patients. These parameters might prove to be a consistent finding in depression. In addition, peripheral DNA methylation of the *MORC1* gene promoter showed stable associations with depression across independent samples. However, the question arises whether *MORC1*, supposedly acting as transcription factor, might also be involved in neurobiological alterations accompanying depression. This study further analyses the role of *MORC1* in depression by investigating a potential correlation between peripheral *MORC1* DNA methylation and neuronal structural properties previously associated with depression in humans.

Methods: Beck Depression Inventory (BDI) was assessed in 52 healthy participants. DNA was extracted from buccal cells and *MORC1* methylation correlated with micro- and macrostructural properties derived from magnetic resonance imaging (MRI) and neurite orientation dispersion and density imaging (NODDI) in the hippocampus and medial prefrontal cortex (mPFC).

Results: *MORC1* methylation was associated with volume reduction and neurite orientation dispersion and density markers in the hippocampus and mPFC. BDI was positively associated with neurite orientation dispersion and density markers in the hippocampus.

Limitations: The study was conducted in a small sample of healthy participants with subclinical depressive symptoms. Peripheral tissue was analyzed.

Conclusions: We found significant negative associations between peripheral *MORC1* methylation and macro- and microstructural markers in the hippocampus and mPFC. Thus, *MORC1* might be involved in neurobiological properties. Studies investigating neuronal methylation patterns of *MORC1* are needed to support this hypothesis

Keywords depression, multi shell DWI, NODDI, grey matter, IMAGE-CpG, epigenetics

1. Introduction

More than 300 million people are suffering from Major Depressive Disorder (MDD) worldwide (World Health Organization, 2018). In the global burden of disease study across 188 countries, MDD was within the top ten leading causes for years lived with disability in every country (Global Burden of Disease Study 2013 Collaborators, 2015). So far, multifactorial origins have been determined for MDD, including environmental factors as well as genetic predispositions (World Health Organization, 2018). Researchers have been trying to enable early detection and understand the pathological alterations accompanying MDD patients on all possible levels. Besides genetic variation investigated in genome-wide association studies (GWAS) (Aragam et al., 2011; Sullivan et al., 2009) and epigenetic changes of DNA packing (Farrell and O'Keane, 2016) structural as well as functional changes in the brain are also associated with MDD (Arnone, 2019; Belleau et al., 2019).

In MDD patients, volume reductions have been most profound in the hippocampus and prefrontal cortex (PFC) and could be linked to symptom severity (Belleau et al., 2019). Besides volume reduction, decreased functional activity or reduced neuron density are also described for some regions in MDD patients (Grimm et al., 2008; Santos et al., 2018; Uranova et al., 2004). Hippocampus volume, for example, is found to be significantly reduced in MDD patients compared to healthy controls in multiple studies (Malykhin et al., 2010; Videbech and Ravnkilde, 2004; Zhao et al., 2017). A meta-analysis of 29 magnetic resonance imaging (MRI) studies including over 1300 patients and 1000 controls found significant hippocampus volume atrophy even though the studies were highly heterogeneous regarding age, sex, age of onset and illness duration (Santos et al., 2018) indicating a strong link between reduced hippocampus volume and depression. This association was already found in a previous meta-analysis of MRI studies in depressive and bipolar patients (Videbech and Ravnkilde, 2004). Another study investigating early-onset depression and hippocampus volume with MRI in a small sample found that hippocampal volume correlated negatively with age of onset of depression, with

more reduction in the left hemisphere (MacMaster and Kusumakar, 2004). Brain activity measuring blood flow or energy metabolism can also be observed using functional MRI (fMRI). A study analyzing fMRI while viewing emotional pictures found hypoactivity in regions of PFC, temporal and parietal cortex, insula, hippocampus, and basal ganglia in MDD patients compared to controls. Interestingly, this hypoactivity could be aligned after antidepressant treatment (Schaefer et al., 2006).

In a post mortem electron microscopy study in the PFC, a significant reduction of oligodendrocyte density was found in MDD, bipolar disorder, and schizophrenia patients compared to controls (Uranova et al., 2004). Regarding the involvement of the PFC, a study using fMRI with medication-free MDD patients revealed less activity in the left dorsolateral (dl) PFC during an emotional judgment task but hyperactivity in the right dlPFC compared to healthy controls indicating a left-right dlPFC imbalance (Grimm et al., 2008). Microstructural alterations can also be investigated using diffusion-weighted imaging (DWI). A new DWI technique to investigate the in vivo microstructural brain architecture is neurite orientation dispersion and density imaging (NODDI) (Zhang et al., 2012). This method allows the quantitative investigation of neurite morphology based on a multi-shell high-angular-resolution diffusion imaging protocol thus enabling the analysis of diffusion-weighted data regarding the microstructure in the gray and white matter (Jespersen et al., 2010, 2007). Using NODDI, current studies have investigated, for example, interindividual differences in cognitive ability (Genç et al., 2018), language processing (Ocklenburg et al., 2018), hemispheric asymmetries (Schmitz et al., 2019a) as well as alterations in MDD (Ota et al., 2018). Analyzing DWI changes in MDD patients revealed multiple asymmetrical alterations such as anisotropy reduction and neurite density reduction in cortical regions as well as in thalamic and parahippocampal regions, so far (Ota et al., 2018). Moreover, another DWI study in MDD patients and matched healthy controls revealed reduced subfield connectivity in the left hippocampus in MDD patients as well as a positive correlation between subfield connectivity and age of onset of depression

(Rutland et al., 2019). However, studies using DWI to investigate microstructural changes in MDD are still rare and have rather small samples sizes. Given all reported alterations in different brain regions in MDD, it is still unclear how exactly these alterations arise. A possible explanation would be altered neuronal gene expression leading to reduced neurite density and thus resulting in reduced volume and connectivity.

Recently, the *MORC family CW-type zinc finger 1* (*MORC1*) gene has attracted increasing attention for being a possible biomarker for depression (Mundorf et al., 2018; Nieratschker et al., 2014; Thomas et al., 2020). Nieratschker and colleagues found altered *MORC1* methylation being associated with early life stress (ELS) in a cross-species study including human cord blood, primate blood and rat brain tissue (Nieratschker et al., 2014). Moreover, they were the first to report an association between certain genetic variations of *MORC1* and MDD (Nieratschker et al., 2014). In a study investigating a *Morc1* knockout, mice lacking the *Morc1* gene showed depressive-like behavior in the forced swim test (Schmidt et al., 2016). This link between reduced *Morc1* expression and depressive symptoms could be validated in healthy humans. There, we found an association between increased *MORC1* methylation in buccal cells and increased depressive symptoms (measured with the Beck Depression Inventory; BDI) (Mundorf et al., 2018). Lastly, in a multicentric study investigating *MORC1* methylation in whole blood cells, childhood trauma and depression in depressive patients and healthy controls, no association between methylation and childhood trauma were found but again, the association between depressive symptoms and *MORC1* methylation was significant in all three cohorts investigated (Thomas et al., 2020).

Besides the validity of *MORC1* methylation as a biomarker for MDD the question arises whether *MORC1*, potentially acting as a transcription factor (Perry and Zhao, 2003), might also be associated with macro- and microstructural brain properties. The present study therefore expands our previous study investigating *MORC1* methylation and subclinical depressive symptoms by correlating *MORC1* methylation and macro- and microstructural properties in the

medial (m) PFC and hippocampus from 52 healthy participants. We therewith investigated whether *MORC1* might also be involved in neurobiological alterations accompanying depression.

2. Methods

Sample

We tested a cohort of 52 healthy adults of German descent (25 females, M_{age} = 24.27, SD_{age} = 2.95). Participants were genetically unrelated and free from psychiatric or neurological disorders as determined by self-report. Depressive symptoms were assessed using the Beck Depression Inventory (BDI) (Beck et al., 1996). All participants gave written informed consent and were treated in accordance with the Declaration of Helsinki. The study was approved by the ethics committee of the Faculty of Psychology, Ruhr University Bochum.

DNA methylation

DNA was collected from buccal swabs and isolated using the blackPREP Swab DNA Kit (Analytik Jena, Germany). Bisulfite conversion was performed using the EpiTect Kit (Qiagen, Germany). Bisulfite-converted DNA was processed on the Illumina MethylationEPIC array (Illumina, United States). Quality control, preprocessing and processing were performed using RStudio version 0.99.903 (RStudio, Inc., United States) and RnBeads (Max Planck Institute for Informatics, Germany) (Assenov et al., 2014) as described previously (Schmitz et al., 2018). The Illumina MethylationEPIC array covered 13 CGsites in the *MORC1* promoter region, defined as 1500 bp upstream and 500 bp downstream of the TSS (chr3: 108836490-108838489) (Mundorf et al., 2018).

Neuroimaging

Acquisition: All neuroimaging data were acquired at the Bergmannsheil hospital in Bochum, Germany, using a Philips 3T Achieva scanner (Best, The Netherlands) with a 32-channel head coil.

Anatomical imaging: We acquired T1-weighted high-resolution anatomical images (MP-RAGE, TR (repetition time) = 8.2 ms, TE (echo time) = 3.7 ms, flip angle = 8°, 220 slices, matrix size = 240 × 240, voxel size = 1 × 1 × 1 mm).

Diffusion-weighted imaging: Diffusion-weighted images were acquired using echo planar imaging (TR = 7652 ms, TE = 87 ms, flip angle = 90°, 60 slices, matrix size = 112 × 112, voxel size = 2 × 2 × 2 mm). We employed a multi-shell, high-angular-resolution scheme using diffusion-weighted images with *b*-values of 1000, 1800, and 2500 s/mm^2, applied along 20, 40, and 60 uniformly distributed directions. Diffusion directions within and between shells were generated orthogonal to each other using the MASSIVE toolbox (Froeling et al., 2017). Eight volumes were acquired with no diffusion weighting (b = 0 s/mm²) for anatomical reference for motion correction and computation of NODDI coefficients.

Analyis of anatomical data: We used FreeSurfer version 5.3.0 (http://surfer.nmr.mgh.harvard.edu) to reconstruct the cortical surfaces of T1-weighted images (Dale et al., 1999; Fischl et al., 1999). The automatic reconstruction steps included skull stripping, gray and white matter segmentation and cortical surface reconstruction and inflation, individually performed for each participant. Each segmentation slice was checked for inaccuracies and manually edited if necessary. Hippocampus and mPFC were parcellated per hemisphere from the T1-weighted images using an automated segmentation procedure based on the Desikan atlas (Desikan et al., 2006) implemented in FreeSurfer. Hippocampus and mPFC were linearly transformed into the native space of the diffucion-weighted images to serve as anatomical landmarks for the extraction of NODDI coefficients.

Analysis of diffusion data: Preprocessing of diffusion images was performed using FMRIB's Diffusion Toolbox (FDT) in FMRIB Software Library (FSL) version 5.0.7. Preprocessing was performed as described previously (Genç et al., 2018; Schmitz et al., 2019b). NODDI coefficients were computed using the AMICO toolbox (Daducci et al., 2015). NODDI

features a three-compartment model distinguishing intra-neurite, extra-neurite, and cerebrospinal environments. First, the proportion of free moving water within each voxel is estimated (isotropic volume fraction, ISO), reflecting isotropic diffusion with Gaussian properties likely to be found in the cerebrospinal fluid. The remaining portion of the signal is divided into the intra-neurite volume fraction (INVF) and neurite orientation dispersion (ODI) (Zhang et al., 2012). For a detailed description of NODDI coefficients and their histological validation see (Grussu et al., 2017; Jespersen et al., 2010, 2007; Zhang et al., 2012). As described above, hippocampus and mPFC were transformed into the native space of the diffusion-weighted images to compute voxel-wise NODDI coefficients (INVF, ODI, ISO).

Statistical analysis

As previous studies suggest a differential effect of the left and right hippocampus (MacMaster and Kusumakar, 2004), macro- and microstructural properties of the hippocampus were analysed for the left and right hemisphere separately, resulting in eight variables (INVF, ODI, ISO, and volume; for the left and right hemisphere).

For the mPFC, there was no indication for an effect of hemispheric asymmetry and average values over both hemispheres were analysed. This resulted in six variables (INVF, ODI, ISO, volume, surface, thickness).

Bivariate Pearson correlations were performed to investigate the relationship between BDI score, DNA methylation levels at the 13 CGsites as well as averaged promoter region (15 variables) with macro- and microstructural properties of the hippocampus (8 variables) and mPFC (6 variables). To adjust for multiple comparisons, FDR correction was applied for hippocampus and mPFC, respectively.

As we previously reported a sex effect on DNA methylation in cg07090057 (Mundorf et al., 2018), we tested for a moderating effect of sex on the left and right hippocampus INVF in a linear regression model.

Statistical analysis and data visualization were performed using RStudio version 1.2.1335 with R 3.5.3.

3. Results

MORC1 DNA methylation and hippocampus micro- and macrostructure

Microstructure: The overall *MORC1* promoter region was not significantly correlated with either microstructural feature (INVF, ODI, ISO) (all $p > .05$). Among the individual CGsites, cg19748686 was nominally significantly correlated with ISO in the left hippocampus ($r = 0.315$, $p = .023$) (see Figure 1A).

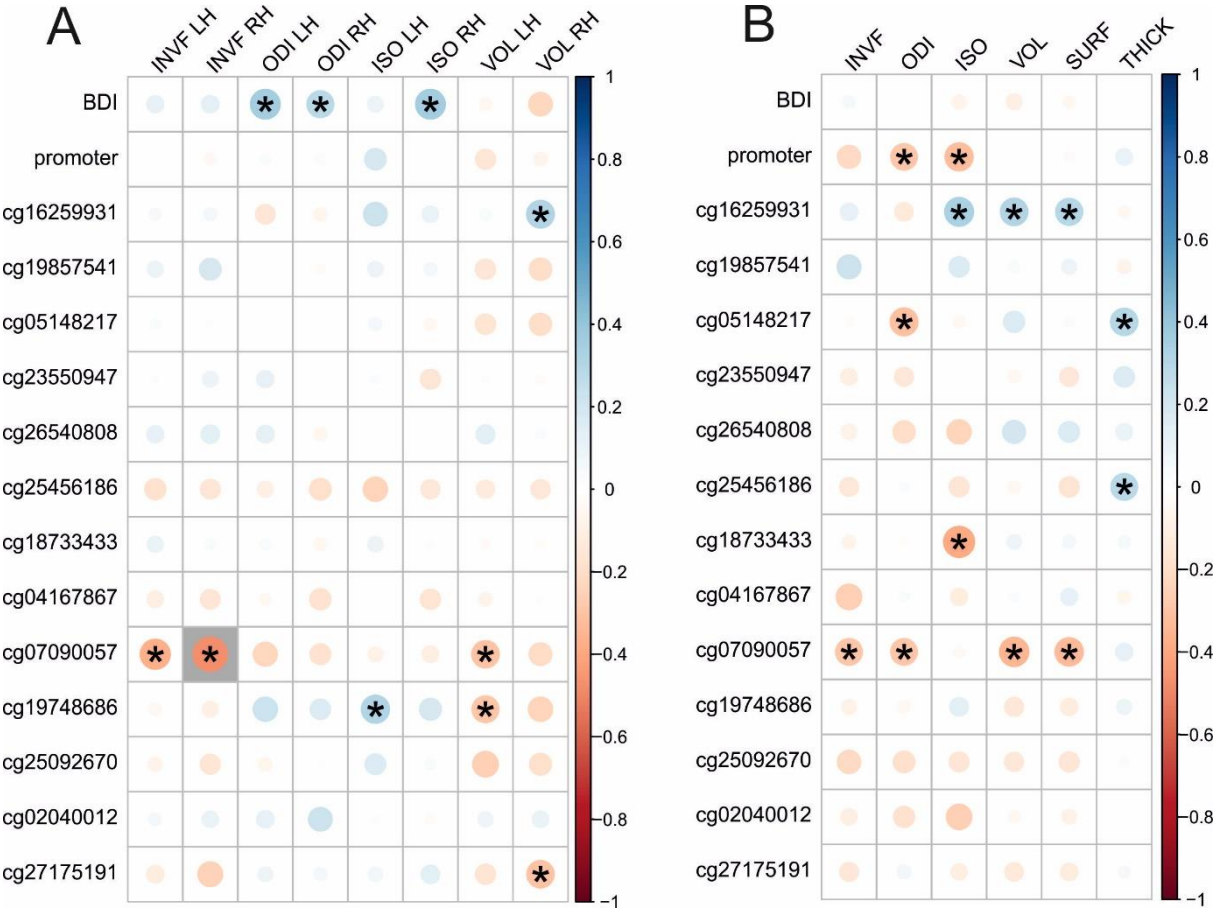


Figure 1: Correlations between BDI and *MORC1* DNA methylation with micro- and macrostructural properties of the hippocampus (A) and mPFC (B). *nominal significance $p < .05$, grey square: FDR-corrected significance

The strongest effect was a significant negative correlation between cg07090057 and left- (r = -0.361, p = .009) as well as right-hemispheric (r = -0.472, p = .0004) hippocampal INVF (see Figure 2).

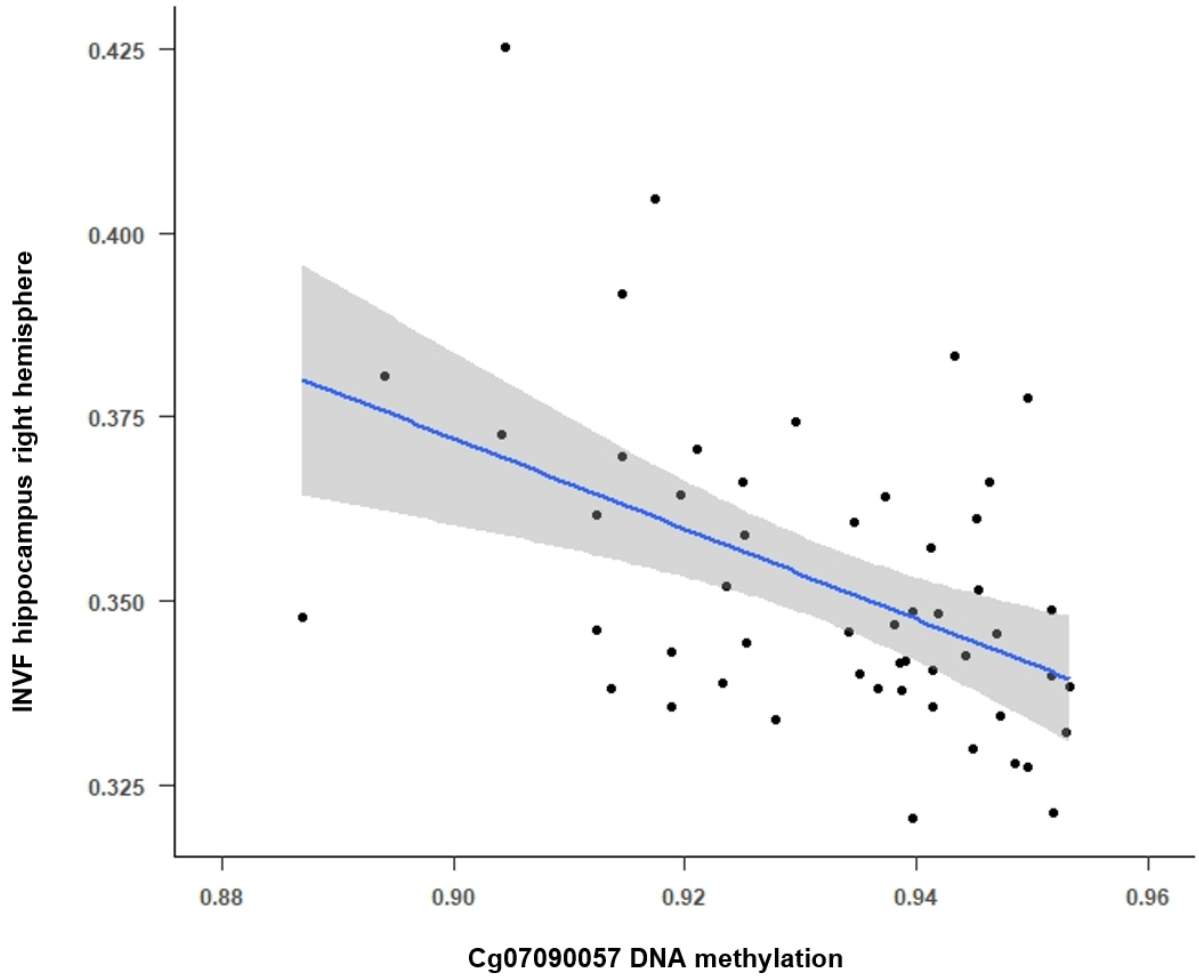


Figure 2: Scatterplot showing the association between cg07090057 DNA methylation and INVF in the right hippocampus.

Macrostructure: The overall *MORC1* promoter region was not significantly correlated with left- or right-hemispheric hippocampal volume (both p > .05). Cg16259931 and cg27175191 showed a nominal significant correlation with right hippocampal volume (cg16259931: r = 0.307, p = .027; cg27175191: r = -0.300, p = .031). Cg07090057 and cg19748686 showed a

nominal significant correlation with left hippocampal volume (cg07090057: r = -0.299, p = .031; cg19748686: r = -0.287, p = .039).

MORC1 DNA methylation and mPFC micro- and macrostructure

Microstructure: The overall *MORC1* promoter region showed a nominally significant correlation with mPFC ODI (r = -0.280, p = .044) and ISO (r = -0.316, p = .023). For ODI, this effect was based on nominally significant correlations of cg05148217 (r = -0.310, p = .026) and cg07090057 (r = -0.282, p = .043) with mPFC ODI. For ISO, the overall effect of the promoter region was based on a significant correlation of cg18733433 (r = -0.385, p = .005) with mPFC ISO, while cg16259931 showed the opposite trend (r = 0.331, p = .016). cg07090057 was also negatively correlated with mPFC INVF (r = -0.285, p = .041) (see Figure 1B).

Macrostructure: The overall *MORC1* promoter region was not significantly correlated with mPFC volume, surface or thickness (all p > .05). Among the individual CG sites, cg16259931 was positively correlated with mPFC volume (r = 0.303, p = .029) and surface (r = 0.291, p = .036), while cg05148217 (r = 0.298, p = .032) and cg25456186 (r = 0.286, p = .040) were positively correlated with mPFC thickness. Cg07090057 was negatively correlated with mPFC volume (r = -0.342, p = .013) and surface (r = -0.320, p = .021).

BDI scores and hippocampus and mPFC micro- and macrostructure

BDI scores were not significantly correlated with INVF in the left or right hippocampus (both p > .05). However, BDI scores showed a nominally significant positive correlation with ODI in the left (r = 0.353, p = .010) and right hippocampus (r = 0.288, p = .039) as well as ISO in the right hippocampus (r = 0.353, p = .010) (see Figure 1A). BDI scores were not significantly correlated with hippocampal volume (both p > .05, see Figure 1A) or mPFC micro- or macrostructure (all p > .05) (see Figure 1B). For all correlation coefficients and p values, see supplementary tables S1 and S2.

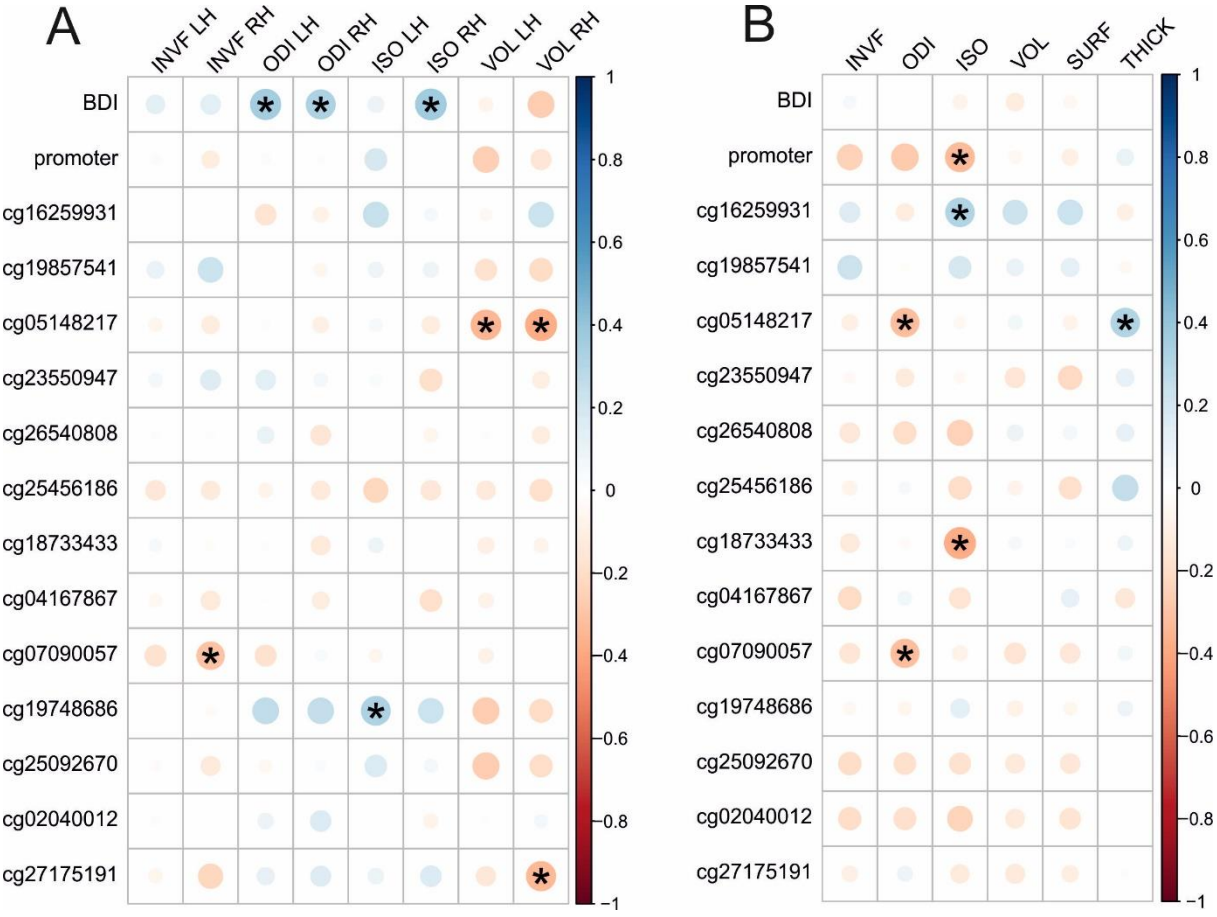


Figure 3: Sex- and age-adjusted partial correlations between BDI and *MORC1* DNA methylation with micro- and macrostructural properties of the hippocampus (A) and mPFC (B). *nominal significance $p < .05$

Moderator analysis

Based on the sex effect on DNA methylation in cg07090057, we tested whether sex moderated the association between cg07090057 and INVF in the left and right hippocampus by running a linear regression including the interaction term. There was no evidence for a moderating effect of sex for INVF in the left ($ß_{interaction}$ = -0.377, p = .336) nor in the right hippocampus ($ß_{interaction}$ = -0.102, p = .733).

4. Discussion

We reported nominal significant associations of *MORC1* methylation and macrostructural features as well as different neurite density, neurite orientation dispersion, and cerebrospinal properties in the hippocampus and mPFC of healthy participants. Interestingly, self-rated BDI scores correlated positively with left and right hippocampus ODI and right hippocampus ISO.

Regarding the *MORC1* promotor region methylation, increased methylation of cg07090057 was associated with multiple macro- and microstructural features of the hippocampus and mPFC. Other CGsites of the promotor region were nominally associated with different features. Still, the prominent and repeatedly negative association of cg07090057 marks this site as potentially most important or most sensitive regarding altered methylation and brain structure. Besides negative associations, positive associations of cg16259931 with hippocampal (volume right) and mPFC (ISO, volume and surface) features were found. However, whether the CGsites have different biological functions regarding *MORC1* expression is still unknown. The found nominal positive correlation between self-rated BDI scores and hippocampus ODI and ISO might be indicative of a structural more distinct hippocampus in participants that reported to be more depressive. Unfortunately, this study does not render enough information to further interpret this result at this point. Of interest, we reported a significant positive association between *MORC1* methylation and BDI in our previous study (Mundorf et al., 2018). The current study is conducted with a subset of the previous sample (52 instead of 60 participants) and the previously reported association is still significant in this subset.

In the hippocampus, increased cg07090057 methylation was associated with decreased INVF in the left and right hemisphere as well as with a reduction in volume in the left hemisphere. Reduced hippocampus volume has previously been described in depressed patients (MacMaster and Kusumakar, 2004). More precisely, in an MRI study with early-onset depression patients (13-18 years of age) a reduced volume in the left hippocampus was found (MacMaster and Kusumakar, 2004). Moreover, hippocampus volume correlated negatively with the age of onset meaning a smaller hippocampus volume with increasing years after the first diagnosis (MacMaster and Kusumakar, 2004). Thus, the effect might be less severe in young and subclinical people. Reduced subfield connectivity in the left hippocampus was also found in MDD patients (Rutland et al., 2019). Again, subfield connectivity and age of onset of depression correlated positively (Rutland et al., 2019). In the mPFC, increased cg07090057

methylation was associated with decreased INVF and ODI as well as reduced volume and thickness. Decreased ODI and INVF might be early signs of the previously identified reduction of oligodendrocytes PFC density found in MDD patients (Uranova et al., 2004). As oligodendrocytes myelinate neurons, reduces neuronal density would also lead to a reduced number of oligodendrocytes.

Generally, INVF gives an estimate of gray matter dendrite and axonal density proving to be negatively related to cell body density (Jespersen et al., 2007). By using stereological analysis, a strong correlation between INVF and density of myelinated axons ($r = 0.97$) was found indicating that INVF is a marker of myelinated axonal density (Jespersen et al., 2010). With INVF as a marker of neurite density it can be assumed that the here found reduced INVF indicates reduced neurite density which might reflect less neurons. Given the here found association between a higher degree of *MORC1* methylation and reduced neurite density it can be hypothesized that *MORC1* might be involved in pathways leading to less neurons. Of note, a higher degree of methylation is frequently associated with less gene activity resulting in less expression (Razin and Cedar, 1991; Razin and Riggs, 1980). Thus, increased *MORC1* methylation might result in reduced gene expression which might lead to reduced NODDI signals. The MORC1 protein is described to have a CW-zing finger protein domain which can be related to chromatin methylation status (Perry and Zhao, 2003) and thus, holds the potential to influence transcription. But studies investigating brain DNA methylation are needed to further investigate this association.

Generally, the found volume reductions might be mediated by stress. It is hypothesized that every new stress exposure or depressive episode activates neurotoxic pathways leading to a decline of brain structures with the progress of the disorder (Belleau et al., 2019). As we investigated healthy participants, with some of them reporting subclinical depressive symptoms, the here found decrease in volume and NODDI markers might be an early detection

of induced alterations. Possibly, the associations between *MORC1* methylation and macro- and microstructural features will be stronger in clinically depressed patients.

Also, the found asymmetrical hemispheric alterations are in line with previous studies regarding general hemispheric functioning. Multiple studies already have reported hemispherical lateralization of specific functions such as emotion processing (Davidson, 1992; Rutherford and Lindell, 2011; Silberman and Weingartner, 1986). Lesion studies in humans revealed that the left frontal hemisphere controls positive and the right frontal hemisphere negative emotions (Rutherford and Lindell, 2011). Damage to one hemisphere leads to reduced respective emotionality and an asymmetrical control of emotions (Rutherford and Lindell, 2011). This hypothesis of hemispheric lateralization of emotion is called valence hypothesis (Silberman and Weingartner, 1986). Regarding this principle, the here found negative correlation of cg07090057 methylation and INVF in the right hippocampus might be responsible for lower activity thus less positive memory processing.

So far, *MORC1* methylation has been linked to depressive symptoms in subclinical and clinical participants (Mundorf et al., 2018; Thomas et al., 2020). Single nucleotide polymorphisms in the DNA sequence of *MORC1* have been associated with MDD (Nieratschker et al., 2014) as well as a gene knockout with depressive behavior in mice (Schmidt et al., 2016). Finding associations between peripheral *MORC1* methylation and reduced volume and neurite morphology in key regions in the brain reinforces the potential role of *MORC1* in depression. More studies analyzing different peripheral and neuronal markers of depression would be helpful in advancing disentangling possible etiologies of depression. As *MORC1* potentially acts as a transcription factor it holds the potential to be involved in stress-regulated pathways leading to alterations found in depression.

Limitations

As we performed multiple statistical analyses, the results should be interpreted carefully. Moreover, the sample size is rather low (52 participants), does not include clinically diagnosed depressed patients and significant results are not corrected for FDR. However, we believe that this data set does render important insights into the function and involvement of *MORC1* in the development of depression. Further studies conducted with clinically depressed patients and larger sample sizes are needed to strengthen this finding.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contribution

A.M. and J.S. designed the study and wrote the manuscript. All authors revised the manuscript. C.S., C.F. and J.S. collected data and were supported by K.H. E.G., C.F., and J.S. analyzed the data. All authors approved the manuscript. This manuscript is our original work and it is submitted for first publication.

Abbreviations

BDI: Beck Depression Inventory
dl: dorsolateral
DWI: Diffusion-weighted imaging
ELS: Early life stress
fMRI: Functional magnetic resonance imaging
GWAS: Genome-wide association study
INVF: Intra-neurite volume fraction
ISO: Isotropic volume fraction
mPFC: medial prefrontal cortex
MDD: Major depressive disorder
MORC1: *MORC family CW-type zinc finger 1*
MRI: Magnetic resonance imaging
NODDI: Neurite orientation dispersion and density imaging
ODI: Neurite orientation dispersion
PFC: Prefrontal cortex

References

Aragam, N., Wang, K.-S., Pan, Y., 2011. Genome-wide association analysis of gender differences in major depressive disorder in the Netherlands NESDA and NTR population-based samples. J. Affect. Disord. 133, 516–521. https://doi.org/10.1016/j.jad.2011.04.054

Arnone, D., 2019. Functional MRI findings, pharmacological treatment in major depression and clinical response. Prog. Neuropsychopharmacol. Biol. Psychiatry 91, 28–37. https://doi.org/10.1016/j.pnpbp.2018.08.004

Assenov, Y., Müller, F., Lutsik, P., Walter, J., Lengauer, T., Bock, C., 2014. Comprehensive analysis of DNA methylation data with RnBeads. Nat. Methods 11, 1138–1140. https://doi.org/10.1038/nmeth.3115

Beck, A., Steer, R.A., Brown, G.K., 1996. Beck Depression Inventory – Second Edition: Manual. Psychol. Corp. San Antonio TX.

Belleau, E.L., Treadway, M.T., Pizzagalli, D.A., 2019. The Impact of Stress and Major Depressive Disorder on Hippocampal and Medial Prefrontal Cortex Morphology. Biol. Psychiatry 85, 443–453. https://doi.org/10.1016/j.biopsych.2018.09.031

Daducci, A., Canales-Rodríguez, E.J., Zhang, H., Dyrby, T.B., Alexander, D.C., Thiran, J.-P., 2015. Accelerated Microstructure Imaging via Convex Optimization (AMICO) from diffusion MRI data. NeuroImage 105, 32–44. https://doi.org/10.1016/j.neuroimage.2014.10.026

Dale, A.M., Fischl, B., Sereno, M.I., 1999. Cortical Surface-Based Analysis: I. Segmentation and Surface Reconstruction. NeuroImage 9, 179–194. https://doi.org/10.1006/nimg.1998.0395

Davidson, R.J., 1992. Anterior cerebral asymmetry and the nature of emotion. Brain Cogn. 20, 125–151. https://doi.org/10.1016/0278-2626(92)90065-t

Desikan, R.S., Ségonne, F., Fischl, B., Quinn, B.T., Dickerson, B.C., Blacker, D., Buckner, R.L., Dale, A.M., Maguire, R.P., Hyman, B.T., Albert, M.S., Killiany, R.J., 2006. An automated labeling system for subdividing the human cerebral cortex on MRI scans into gyral based regions of interest. NeuroImage 31, 968–980. https://doi.org/10.1016/j.neuroimage.2006.01.021

Farrell, C., O'Keane, V., 2016. Epigenetics and the glucocorticoid receptor: A review of the implications in depression. Psychiatry Res. 242, 349–356. https://doi.org/10.1016/j.psychres.2016.06.022

Fischl, B., Sereno, M.I., Dale, A.M., 1999. Cortical Surface-Based Analysis: II: Inflation, Flattening, and a Surface-Based Coordinate System. NeuroImage 9, 195–207. https://doi.org/10.1006/nimg.1998.0396

Froeling, M., Tax, C.M.W., Vos, S.B., Luijten, P.R., Leemans, A., 2017. "MASSIVE" brain dataset: Multiple acquisitions for standardization of structural imaging validation and evaluation. Magn. Reson. Med. 77, 1797–1809. https://doi.org/10.1002/mrm.26259

Genç, E., Fraenz, C., Schlüter, C., Friedrich, P., Hossiep, R., Voelkle, M.C., Ling, J.M., Güntürkün, O., Jung, R.E., 2018. Diffusion markers of dendritic density and arborization in gray matter predict differences in intelligence. Nat. Commun. 9, 1–11. https://doi.org/10.1038/s41467-018-04268-8

Global Burden of Disease Study 2013 Collaborators, 2015. Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: a systematic analysis for the Global Burden of Disease

Study 2013. Lancet Lond. Engl. 386, 743–800. https://doi.org/10.1016/S0140-6736(15)60692-4

Grimm, S., Beck, J., Schuepbach, D., Hell, D., Boesiger, P., Bermpohl, F., Niehaus, L., Boeker, H., Northoff, G., 2008. Imbalance between Left and Right Dorsolateral Prefrontal Cortex in Major Depression Is Linked to Negative Emotional Judgment: An fMRI Study in Severe Major Depressive Disorder. Biol. Psychiatry, Stress, Depression, and Circuitry 63, 369–376. https://doi.org/10.1016/j.biopsych.2007.05.033

Grussu, F., Schneider, T., Tur, C., Yates, R.L., Tachrount, M., Ianuş, A., Yiannakas, M.C., Newcombe, J., Zhang, H., Alexander, D.C., DeLuca, G.C., Gandini Wheeler-Kingshott, C.A.M., 2017. Neurite dispersion: a new marker of multiple sclerosis spinal cord pathology? Ann. Clin. Transl. Neurol. 4, 663–679. https://doi.org/10.1002/acn3.445

Hannon, E., Lunnon, K., Schalkwyk, L., Mill, J., 2015. Interindividual methylomic variation across blood, cortex, and cerebellum: implications for epigenetic studies of neurological and neuropsychiatric phenotypes. Epigenetics 10, 1024–1032. https://doi.org/10.1080/15592294.2015.1100786

Jespersen, S.N., Bjarkam, C.R., Nyengaard, J.R., Chakravarty, M.M., Hansen, B., Vosegaard, T., Østergaard, L., Yablonskiy, D., Nielsen, N.C., Vestergaard-Poulsen, P., 2010. Neurite density from magnetic resonance diffusion measurements at ultrahigh field: comparison with light microscopy and electron microscopy. NeuroImage 49, 205–216. https://doi.org/10.1016/j.neuroimage.2009.08.053

Jespersen, S.N., Kroenke, C.D., Østergaard, L., Ackerman, J.J.H., Yablonskiy, D.A., 2007. Modeling dendrite density from magnetic resonance diffusion measurements. NeuroImage 34, 1473–1486. https://doi.org/10.1016/j.neuroimage.2006.10.037

MacMaster, F.P., Kusumakar, V., 2004. Hippocampal volume in early onset depression. BMC Med. 2, 2. https://doi.org/10.1186/1741-7015-2-2

Malykhin, N.V., Carter, R., Seres, P., Coupland, N.J., 2010. Structural changes in the hippocampus in major depressive disorder: contributions of disease and treatment. J. Psychiatry Neurosci. JPN 35, 337–343. https://doi.org/10.1503/jpn.100002

Mundorf, A., Schmitz, J., Güntürkün, O., Freund, N., Ocklenburg, S., 2018. Methylation of MORC1: A possible biomarker for depression? J. Psychiatr. Res. 103, 208–211. https://doi.org/10.1016/j.jpsychires.2018.05.026

Nieratschker, V., Massart, R., Gilles, M., Luoni, A., Suderman, M.J., Krumm, B., Meier, S., Witt, S.H., Nöthen, M.M., Suomi, S.J., Peus, V., Scharnholz, B., Dukal, H., Hohmeyer, C., Wolf, I. a.-C., Cirulli, F., Gass, P., Sütterlin, M.W., Filsinger, B., Laucht, M., Riva, M.A., Rietschel, M., Deuschle, M., Szyf, M., 2014. MORC1 exhibits cross-species differential methylation in association with early life stress as well as genome-wide association with MDD. Transl. Psychiatry 4, e429. https://doi.org/10.1038/tp.2014.75

Ocklenburg, S., Friedrich, P., Fraenz, C., Schlüter, C., Beste, C., Güntürkün, O., Genç, E., 2018. Neurite architecture of the planum temporale predicts neurophysiological processing of auditory speech. Sci. Adv. 4, eaar6830. https://doi.org/10.1126/sciadv.aar6830

Ota, M., Noda, T., Sato, N., Hidese, S., Teraishi, T., Setoyama, S., Sone, D., Matsuda, H., Kunugi, H., 2018. The use of diffusional kurtosis imaging and neurite orientation dispersion and density imaging of the brain in major depressive disorder. J. Psychiatr. Res. 98, 22–29. https://doi.org/10.1016/j.jpsychires.2017.12.011

Perry, J., Zhao, Y., 2003. The CW domain, a structural module shared amongst vertebrates, vertebrate-infecting parasites and higher plants. Trends Biochem. Sci. 28, 576–580. https://doi.org/10.1016/j.tibs.2003.09.007

Razin, A., Cedar, H., 1991. DNA methylation and gene expression. Microbiol. Rev. 55, 451–458.

Razin, A., Riggs, A.D., 1980. DNA methylation and gene function. Science 210, 604–610.

Rutherford, H.J.V., Lindell, A.K., 2011. Thriving and Surviving: Approach and Avoidance Motivation and Lateralization. Emot. Rev. 3, 333–343. https://doi.org/10.1177/1754073911402392

Rutland, J.W., Brown, S., Verma, G., Feldman, R.E., Sharma, H., Markowitz, M., Schneider, M., Delman, B.N., Murrough, J., Balchandani, P., 2019. Hippocampal subfield-specific connectivity findings in major depressive disorder: A 7 Tesla diffusion MRI study. J. Psychiatr. Res. 111, 186–192. https://doi.org/10.1016/j.jpsychires.2019.02.008

Santos, M.A.O., Bezerra, L.S., Carvalho, A.R.M.R., Brainer-Lima, A.M., 2018. Global hippocampal atrophy in major depressive disorder: a meta-analysis of magnetic resonance imaging studies. Trends Psychiatry Psychother. 40, 369–378. https://doi.org/10.1590/2237-6089-2017-0130

Schaefer, H.S., Putnam, K.M., Benca, R.M., Davidson, R.J., 2006. Event-related functional magnetic resonance imaging measures of neural activity to positive social stimuli in pre- and post-treatment depression. Biol. Psychiatry 60, 974–986. https://doi.org/10.1016/j.biopsych.2006.03.024

Schmidt, M., Brandwein, C., Luoni, A., Sandrini, P., Calzoni, T., Deuschle, M., Cirulli, F., Riva, M.A., Gass, P., 2016. Morc1 knockout evokes a depression-like phenotype in mice. Behav. Brain Res. 296, 7–14. https://doi.org/10.1016/j.bbr.2015.08.005

Schmitz, J., Fraenz, C., Schlüter, C., Friedrich, P., Jung, R.E., Güntürkün, O., Genç, E., Ocklenburg, S., 2019a. Hemispheric asymmetries in cortical gray matter microstructure identified by neurite orientation dispersion and density imaging. NeuroImage 189, 667–675. https://doi.org/10.1016/j.neuroimage.2019.01.079

Schmitz, J., Fraenz, C., Schlüter, C., Friedrich, P., Kumsta, R., Moser, D., Güntürkün, O., Genç, E., Ocklenburg, S., 2019b. Schizotypy and altered hemispheric asymmetries: The role of cilia genes. Psychiatry Res. Neuroimaging 294, 110991. https://doi.org/10.1016/j.pscychresns.2019.110991

Schmitz, J., Kumsta, R., Moser, D., Güntürkün, O., Ocklenburg, S., 2018. KIAA0319 promoter DNA methylation predicts dichotic listening performance in forced-attention conditions. Behav. Brain Res. 337, 1–7. https://doi.org/10.1016/j.bbr.2017.09.035

Silberman, E.K., Weingartner, H., 1986. Hemispheric lateralization of functions related to emotion. Brain Cogn. 5, 322–353. https://doi.org/10.1016/0278-2626(86)90035-7

Sullivan, P.F., de Geus, E.J.C., Willemsen, G., James, M.R., Smit, J.H., Zandbelt, T., Arolt, V., Baune, B.T., Blackwood, D., Cichon, S., Coventry, W.L., Domschke, K., Farmer, A., Fava, M., Gordon, S.D., He, Q., Heath, A.C., Heutink, P., Holsboer, F., Hoogendijk, W.J., Hottenga, J.J., Hu, Y., Kohli, M., Lin, D., Lucae, S., Macintyre, D.J., Maier, W., McGhee, K.A., McGuffin, P., Montgomery, G.W., Muir, W.J., Nolen, W.A., Nöthen, M.M., Perlis, R.H., Pirlo, K., Posthuma, D., Rietschel, M., Rizzu, P., Schosser, A., Smit, A.B., Smoller, J.W., Tzeng, J.-Y., van Dyck, R., Verhage, M., Zitman, F.G., Martin, N.G., Wray, N.R., Boomsma, D.I., Penninx, B.W.J.H., 2009. Genome-wide association for major depressive

disorder: a possible role for the presynaptic protein piccolo. Mol. Psychiatry 14, 359–375. https://doi.org/10.1038/mp.2008.125

Thomas, M., Coope, A., Falkenberg, C., Dunlop, B.W., Czamara, D., Provencal, N., Craighead, W.E., Mayberg, H.S., Nemeroff, C.B., Binder, E.B., Nieratschker, V., 2020. Investigation of MORC1 DNA methylation as biomarker of early life stress and depressive symptoms. J. Psychiatr. Res. 120, 154–162. https://doi.org/10.1016/j.jpsychires.2019.10.019

Uranova, N.A., Vostrikov, V.M., Orlovskaya, D.D., Rachmanova, V.I., 2004. Oligodendroglial density in the prefrontal cortex in schizophrenia and mood disorders: a study from the Stanley Neuropathology Consortium. Schizophr. Res. 67, 269–275. https://doi.org/10.1016/S0920-9964(03)00181-6

Videbech, P., Ravnkilde, B., 2004. Hippocampal volume and depression: a meta-analysis of MRI studies. Am. J. Psychiatry 161, 1957–1966. https://doi.org/10.1176/appi.ajp.161.11.1957

World Health Organization, 2018. WHO | Depression [WWW Document]. WHO. URL http://www.who.int/mediacentre/factsheets/fs369/en/ (accessed 8.29.16).

Zhang, H., Schneider, T., Wheeler-Kingshott, C.A., Alexander, D.C., 2012. NODDI: practical in vivo neurite orientation dispersion and density imaging of the human brain. NeuroImage 61, 1000–1016. https://doi.org/10.1016/j.neuroimage.2012.03.072

Zhao, K., Liu, H., Yan, R., Hua, L., Chen, Y., Shi, J., Lu, Q., Yao, Z., 2017. Cortical thickness and subcortical structure volume abnormalities in patients with major depression with and without anxious symptoms. Brain Behav. 7, e00754. https://doi.org/10.1002/brb3.754

Cigarette smoke exposure has region-specific effects on GDAP1 expression in mouse hippocampus

Annakarina Mundorf [1], Stefanie Rommel [1], Malte Verheyen [2], Evanthia Mergia [2], Marcus Peters [3] and Nadja Freund [1]

[1]Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany

[2]Pharmacology and Toxicology, Ruhr-University Bochum, Germany

[3]Department of Experimental Pneumology, Ruhr-University Bochum, Germany

Mundorf, A, Rommel, S, Verheyen, M, Mergia, E, Peters, M, Freund, N (2020). Cigarette smoke exposure has region-specific effects on GDAP1 expression in mouse hippocampus. Psychiatry Res. July;289:112979, https://doi.org/10.1016/j.psychres.2020.112979

Psychiatry Research 289 (2020) 112979

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Cigarette smoke exposure has region-specific effects on GDAP1 expression in mouse hippocampus

Annakarina Mundorf[a], Stefanie Rommel[a], Malte Verheyen[b], Evanthia Mergia[b], Marcus Peters[c], Nadja Freund[a,*]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Germany
[b] Pharmacology and Toxicology, Ruhr-University Bochum, Germany
[c] Department of Experimental Pneumology, Ruhr-University Bochum, Germany

ARTICLE INFO

Keywords:
Dentate gyrus
CA1
Immunohistochemistry
Mouse model
Nicotinic acetylcholine receptors

ABSTRACT

Early detection markers for substance use disorders are urgently needed. Recently, an association between the methylation of *Ganglioside-induced differentiation-associated protein 1 (GDAP1)* and alcohol addiction was found in a US and German population. In this study, we investigate whether GDAP1 expression might be affected by cigarette smoke as well and thus might be a marker of substance addiction in general. 11 adult female C57BL/6 J mice (6 wildtype and 5 lacking the NO-sensitive guanylyl cyclase1 (NO-GC1 KO)) were exposed to cigarette smoke over a period of 5 weeks, their brains immunohistochemically stained and compared to 11 non exposed mice (5 WT and 6 KO). The deletion of NO-GC1 results in a complete loss of synaptic plasticity, therefore, addiction-related alterations might become more obvious. Co-staining of anti-GDAP1 and DAPI revealed protein in the *stratum granulare* of the hippocampus. Three randomized frames for dentate gyrus (DG) and three for *Cornu Ammonis* region 1 (CA1) were used to count GDAP1. Cigarette smoke exposure significantly influenced GDAP1 expression depending on the hippocampal region but was not influenced by guanyl cyclase. In conclusion, cigarette smoke exposure alone had an effect on GDAP1 amount in both regions. Therewith, *GDAP1*might be a biomarker for substance addiction in general.

1. Introduction

Substance use disorder (SUD) is characterized by mental, behavioral or physical symptoms arising in problems related to loss of control, hazardous use, tolerance, and withdrawal, with addiction being the most severe and chronic stage of SUD (American Psychiatric Association, 2013). In most cases, SUDs such as alcohol use disorder (AUD), are diagnosed (and treated) when frequent and heavy consumption lead to loss of work or break up of relationship or gets the affected person arrested (Philibert et al., 2014). However, a broken social environment hampers integration after a successful inpatient treatment and increases risk of relapse (Walter et al., 2006). Thus, early detection of harmful substance consumption or addiction is urgently needed. Biomarkers can be used to indirectly assess the consequences of consumption on physical health or amount consumed directly (Tavakoli et al., 2011). So far, biomarkers enable to measure the amount consumed but not the dependency or the vulnerability for dependency.

SUD and addiction are discussed to be reinforced by an interaction of genetic and environmental factors, as shown by twin studies investigating the genetic and environmental (peer group) influence on addictive tobacco and other illicit drugs consume (Agrawal et al., 2010; Huizink et al., 2010 for review see Stone et al., 2012). Therefore, it is reasonable to shift the attention of research from genetic to epigenetic alterations. Some studies were able to link DNA methylation alterations in early life to a greater risk for substance use (Cecil et al., 2016) but mostly it is still unclear whether DNA methylation of a gene might lead to addiction or addiction leads to DNA methylation changes (Mahna et al., 2018; Zhang and Gelernter, 2016). Frequently, studies investigating SUD, do not divide between substances, as it might be difficult to separate in humans e.g. most people suffering from AUD are smokers as well (Meyerhoff et al., 2006). Therefore it is no surprise, that some genes are influences by alcohol intake and smoking in the same way. Methylation of the *monoamine oxidase A* (*MAOA*) gene in lymphoblast's, for example, was found to be significantly associated with both, nicotine and alcohol dependence in women (Philibert et al.,

* Corresponding author.
E-mail address: nadja.freund@ruhr-uni-bochum.de (N. Freund).

https://doi.org/10.1016/j.psychres.2020.112979
Received 12 December 2019; Received in revised form 25 March 2020; Accepted 31 March 2020
Available online 07 May 2020

Abbreviations

AUD:	Alcohol use disorder
CA1:	Cornu Ammonis region 1
DAPI:	4′,6-diamidino-2-phenylindole
DG:	Dentate gyrus
GDAP1:	Ganglioside-induced differentiation-associated protein 1
MAOA:	Monoamine oxidase A
nAChR:	nicotinic acetylcholine receptor
NHS:	Normal horse serum
NO-GC1 KO:	NO-sensitive guanylyl cyclase1
PBS:	Phosphate Buffer Saline
SUD:	Substance use disorder
WT:	Wildtype

2008).

Recently, a gene raised attention to be a valid biomarker specifically for AUD. From 29 investigated studies, six out of seven genes were hypermethylated and only *Ganglioside-induced differentiation-associated protein 1 (GDAP1)* was hypomethylated (Zhang and Gelernter, 2016). Reduced methylation of the *GDAP1* promoter region of lymphoblast DNA was associated with alcohol disorder when investigating the effects of alcohol therapy in patients (Philibert et al., 2014). This result could be replicated by Brückmann et al. (2016) in a study with alcohol-dependent male patients. The patients showed less methylation of the promoter region of the *GDAP1* gene in whole blood cells than controls and the degree of hypomethylation was associated with increased alcohol dependence (Brückmann et al., 2016).

But, as mentioned above, frequently people diagnosed with AUD are smokers as well as it was the case in both studies investigating *GDAP1* methylation and AUD (Brückmann et al., 2016; Philibert et al., 2014).

As the association between altered *GDAP1* methylation and AUD was found in an US and a German population and in two different tissues (lymphoblast and whole blood) *GDAP1* methylation holds strong potential as biomarker for AUD. However, the question arises whether *GDAP1* methylation is associated exclusively with alcohol or whether it might also be affected by cigarette smoke and thus might be a biomarker for general substance addiction.

So far, polymorphisms in the *GDAP1* gene were mainly connected to the development of the Charcot-Marie-Tooth-disease which is leading to severe motoric and sensoric neuropathology (Tazir et al., 2014). A lack of GDAP1 protein leads to reduced calcium influx of the cell as well as mitochondrial deficits (BarneoMuñoz et al., 2015). GDAP1 protein is located in the outer membrane of mitochondria and particularly found in Schwann cells and oligodendrocytes (Niemann et al., 2005). Moreover, GDAP1 is expressed by pyramidal neurons of the hippocampus (Pedrola et al., 2008).

In a first approach, we investigated GDAP1 protein amount in mice hippocampus after exposure to cigarette smoke inhalation. We analyzed wildtype (WT) and KO mice lacking the NO-sensitive guanylyl cyclase1 (NO-GC1 KO), which have low cGMP formation upon NO stimulation. NO-GC1 is expressed in the hippocampus and acts as an essential modulator of synaptic transmission in this region. Hence, deletion of NO-GC1 results in a complete loss of synaptic plasticity, i.e. long-term potentiation (LTP) (Koesling et al., 2016). Changes in neuronal plasticity are associated with addiction, so we hypothesized that addiction-related alterations will be more obvious in these KO mice. Cigarette smoke exposure (Holliday et al., 2016) as well as drug abuse including alcohol (for review see Kutlu and Gould, 2016) affects the hippocampus by activation of nicotinic acetylcholine receptors (nAChRs) (for review see Zeid et al., 2018). For example, postmortem studies of smokers revealed increased nAChRs in the hippocampus (e.g. an increase of 290% in the dentate gyrus) as well as in cortical regions compared to nonsmokers (Perry et al., 1999). Another study using brain imaging techniques to investigate further this link between cigarette smoking and nAChRs found that cigarette smoking in typically daily tobacco-dependent smokers leads to nearly complete occupancy of α4β2 nAChRs throughout the day thus leading to desensitization of nAChRs (Brody et al., 2006). NAChRs consist of different α and β subunits which are not homogeneous distributed in the brain. In the rodent hippocampus, mainly α7 and α4β2 nAChR are present, especially in the *striatum granulare* of the dentate gyrus (DG) (John et al., 2015; Kaneko et al., 2006; Zeid et al., 2018). The DG and the *Cornu Ammonis* region 1 (CA1) are known to be involved in memory forming and thus in the development of dependence (Hunsaker et al., 2008; Kesner, 2007).

Biomarkers enabling early detection of substance addiction are needed. In a first approach, we investigate whether *GDAP1,* a marker for AUD, might be a general marker for substance addiction by analyzing GDAP1 expression in the hippocampus of adult mice being exposed to cigarette smoke. The rodent hippocampus might be a target region of cigarette smoke as it is rich in nAChRs which themselves are affected by nicotine. We hypothesize, that GDAP1 might act via increased hippocampus nAChRs.

2. Methods

2.1. Animals

Analysis was performed in the hippocampus region of WT and NO-GC1 KO mice. Adult female mice on a C57BL/6 J background were used between the age of 10 to 20 weeks. Only female mice were used since they are more easily adapted to the restrainer that must be applied for smoke exposure. Animals were group housed in open-top cages on a standard light-dark circle (12/12 hrs, light period 07:00–19:00) with water and food access ad libitum and constant temperature and humidity conditions (22 ± 2 °C and 55 ± 25%).

2.2. Treatment

Mice (6 WT and 5 KO) were exposed to the smoke of five cigarettes (3R4F research cigarettes, University of Kentucky) a day for five weeks. Control (5 WT and 6 KO) mice were exposed to room air. Each cigarette contained 0.73 mg nicotine. The smoke of each cigarette was produced by a fully automatic smoke machine and distributed over 11 mice that were restrained in an inhalation tower to inhale the smoke via the nose only (DSI™, a division of Harvard Bioscience Inc., Saint Paul, United States). The cigarettes were burned with the following smoking characteristics: 4 puffs/min, 40 ml/puff, 3 s/puff. With this smoking profile the cigarettes were burned down to the filter tip in 7 min. After a pause period of 3 min the next cigarette was applied. Smoke concentration was daily measured by photometric analysis and amounted to a mean inhaled concentration of 4 g/m^3. Based on these settings, we expected a blood concentration of at least 2 ng/ml nicotine and 20 ng/ml cotinine as previously measured by others (Phillips et al., 2015). All experiments were carried out in agreement with the principles regarding the care and use of animals adopted by the German Animal Welfare Law for the prevention of cruelty to animals after approval by the LANUV (Landesamt für Natur, Umwelt und Verbraucherschutz Northrhine-Westfalia).

2.3. Immunofluorescence staining

Animals were sacrificed one day after the last exposure, brains were dissected and prepared for staining. After fixation for 2 days with 4% Paraformaldehyde (Roth), the brains were embedded in 3% Agarose Low Melt (Roth) and cut in 40 µm coronal slices with a Vibratome

(Leica VT 1200S). All slices containing the hippocampus (Bregma −0.94 mm to −2.46 mm) were stained according to standard free-floating immunofluorescence protocols (Bachman, 2013) with slide modifications. In brief, slices were blocked for two hours with 10% normal horse serum (NHS) in 0.1% Tween in Phosphate Buffer Saline (PBS). Then, slices were incubated overnight at 4 °C with Anti-GDAP1 out of rabbit (1:200, Sigma-Aldrich, HPA024334) in 0.1% Tween and 1% NHS in PBS. On the next day, after washing slices were incubated in

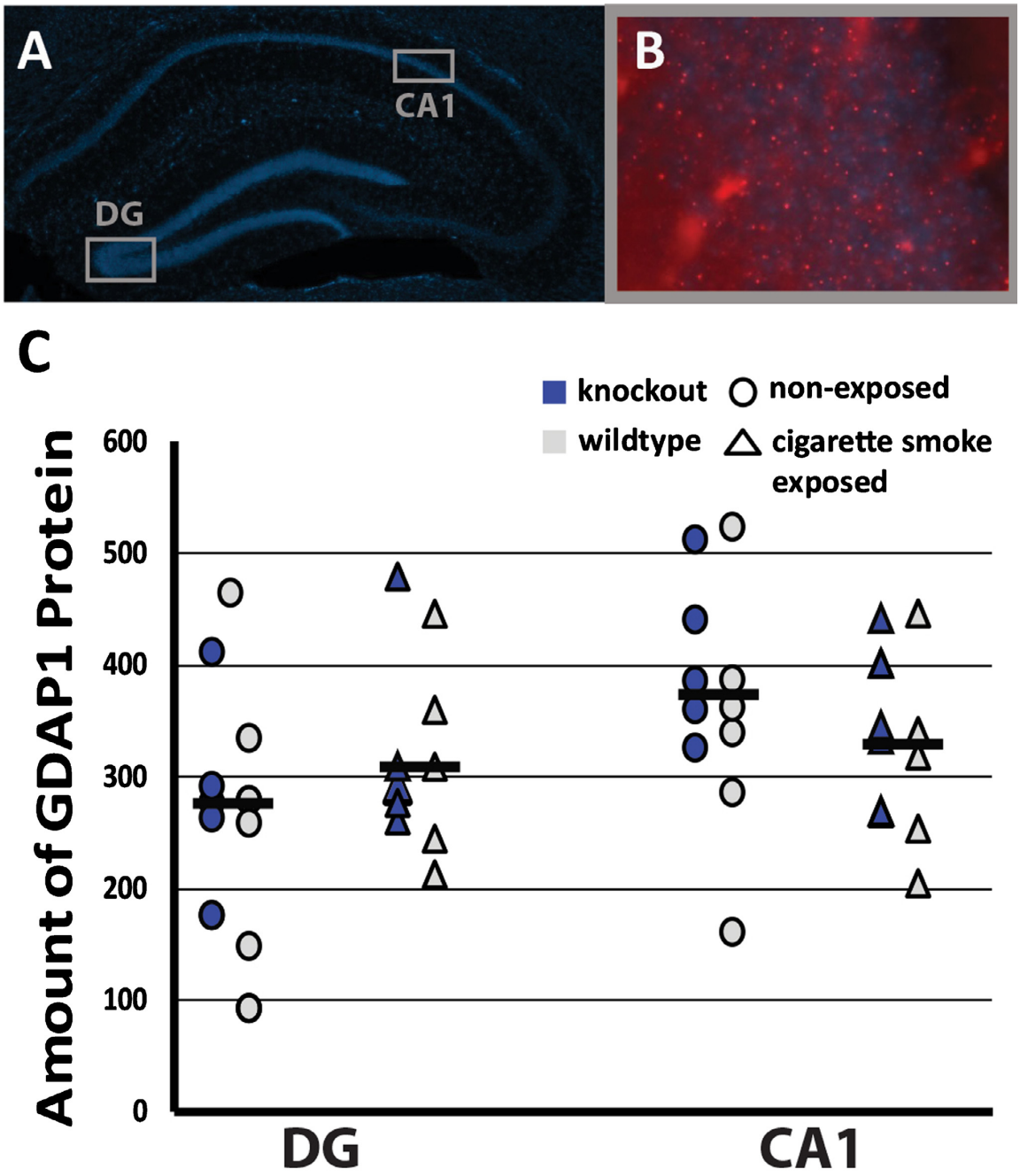


Fig. 1. A: Overview picture of a DAPI stained half Hippocampus in 100 magnifications. gray boxes mark the analyzed areas. **B**: Immunofluorescence co-staining of anti-GDAP1 and DAPI revealed protein staining in the *stratum granulare*. Cells building the *stratum granulare* of the hippocampus are shown in blue (DAPI). GDAP1 Protein (red) and DAPI co-staining is shown in mag.: 400. **C:** Counted amount of GDAP1 in DG and CA1 depending on cigarette smoke exposure and genotype. Non-exposed animals are presented in cycles and cigarette smoke exposed in triangles. Wildtypes are marked in gray and knockouts are represented in blue. Mean is shown as black bars. Exposure leads to more GDAP1 in DG but less GDAP1 in CA1. ANOVA revealed a significant effect of interaction between regions and nicotine exposure.

PBS with 0.1% Tween and Anti-rabbit (1:1000, Sigma-Aldrich, SAB4600068-250UL) for one hour at room temperature. After washing, slices were incubated for 1 min in 5 µg/ml 4′,6-diamidino-2-phenylindole (DAPI) in PBS, then washed, mounted and coverslipped with Mowiol (Roth).

2.4. GDAP1 counting in the stratum granulare

The dentate gyrus was imaged in 400x magnification with the AXIO Imager M1 (Zeiss). Single pictures were merged to obtain an overview image of the whole *stratum granulare* of the dentate gyrus. One hemisphere of the dentate gyrus was divided into 275,40 µm x 198,62 µm frames (1–196). Then, three randomized frames out of the total were analyzed and collapsed into a mean data point for each animal. Whether the left or right hemisphere was imaged was randomly assigned for each frame as well. The CA1 region was imaged as the DG and divided into 275,40 µm x 198,62 µm frames (1–157). Again, three randomized frames out of the total were analyzed and collapsed into a mean data point per animal and region.

The software ImageJ by Fiji was used for counting GDAP1 protein (Rueden et al., 2017; Schindelin et al., 2012). Spherical radius was set to 15 Pixel, upper threshold was set to 25 and lower threshold to 255. The size of the pixelˆ2 was set to 0–4 and circularity to 0,00–1,00.

2.5. Analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics 25). To investigate differences in GDAP1 amount between the two groups, two-factor analysis of variance (ANOVA) with repeated measurement with region (DG and CA1) was performed. Between subject factors were the factor cigarette smoke (exposure or control) and the factor genotype (KO or WT). Bonferroni correction was used for post-hoc analysis.

3. Results

3.1. Immunofluorescence staining

Immunofluorescence co-staining of anti-GDAP1 and DAPI revealed protein staining in the *stratum granulare* of the hippocampus (Fig. 1A+B) as described in the methods.

3.2. Statistics

Two-factor ANOVA with repeated measurements showed a significant effect of region ($F1,18 = 14,707$; $P = 0.001$). Moreover, a significant interaction was found between region x cigarette smoke exposure ($F1,18 = 9178$; $P = 0.007$). However, post-hoc analysis resulted in no significant difference in GDAP1 amount between cigarette smoke exposed and non-exposed animals in the DG region (Student's ttest; $t(20) = -1060$; $P = 0.560$) nor in the CA1 region ($t(20) = 1097$; $P = 0,666$) (Fig. 1C). Genotype had neither a significant effect nor an interaction.

4. Discussion

Over all, cigarette smoke exposure had region dependent effects on GDAP1 expression. While more GDAP1 expression was counted in the DG less could be found in the CA1 region after cigarette smoke exposure (Fig. 1C). No effect of the guanylyl cyclase knockout on GDAP1 expression was found nor had the knockout an influence on the cigarette exposure effects. GDAP1 expression therefore seems independent of NO/cGMP signaling.

Our findings are in line with region-specific effects of nicotine on hippocampus neurons found during developmental periods. For example, regarding CA1, nicotine supply leads to a reduction in length and complexity of dendrites in adolescent mice. However, in adult mice, nicotine affects neurons in the CA3 region but not CA1 region (Holliday et al., 2016).

In the rat DG, nicotine self-administration (Abrous et al., 2002) as well as intraperitoneal injection of nicotine (Shingo and Kito, 2005) decreases neurogenesis and increases cell death. Thus, nicotine can lead to cell death in both regions. Cell death is leading to generally lower amounts of protein in nicotine exposed mice. The harmful effects of nicotine could therefore explain the reduced GDAP1 amount in CA1.

The higher GDAP1 amount in DG after cigarette smoke exposure might be due to neurogenesis. Across different mice strain, C57BL/6 J show the highest proliferation in DG as they produce 0.36% of their total granule cell number as neurons within 6 days (Kempermann et al., 1997). As the mice were exposed to cigarette smoke over a period of 5 weeks it seems likely that neurons proliferated during this time of exposure were influenced by cigarette smoke exposure. So far, alcohol consumption is associated with less methylation of *GDAP1* which in turn is generally associated with more gene expression (Razin and Cedar, 1991). The here found results indicate that cigarette smoke exposure might alter *GDAP1* methylation, in the same way as alcohol consumption did, which is reflected in a higher GDAP1 amount in DG. Neurons proliferated during cigarette smoke exposure might be already born with less *GDAP1* methylation thus express more GDAP1. Therefore, even though neurogenesis might be reduced by cigarette smoke exposure, the few newborn neurons express more GDAP1 leading to a significantly higher amount in DG compared to non-exposed mice.

Higher GDAP1 protein levels might then increase calcium influx in cells as a loss of GDAP1 protein results in less calcium influx (BarneoMuñoz et al., 2015), reinforcing the effect of nicotine. Nicotine induced stimulation of calcium influx might shift the inhibition/excitation balance in the hippocampus. As nicotine application onto hippocampus slices from juvenile rats leads to irregular Ca2+ transients in CA1 region dendrites measured using patch clamp electrophysiology and two-photon laser scanning (Szabo et al., 2008).

Therewith, increased expression of GDAP1 might possibly increase the risk of addiction and craving of substances like nicotine or alcohol. Especially, as nAChRs are known to mediate calcium influx as well (Shen and Yakel, 2009) which themselves can be activated by nicotine and alcohol (Wu et al., 2014). The here found higher amount of GDAP1 in DG after cigarette smoke exposure could therefore be a result of more nAChRs present and consequently reinforce the impact of cigarette smoke which activates hippocampal nAChRs (for review see Zeid et al., 2018).

In conclusion, cigarette smoke exposure leads to more GDAP1 protein in DG and a reduction in CA1 possibly mediated by reduced *GDAP1* methylation. Newborn cells in DG might show reduced *GDAP1* methylation in cigarette smoke exposed mice and thus increase GDAP1 expression. However, these are speculations and need further investigation.

Here we could show that cigarette smoking has an influence on GDAP1 protein expression. Therefore, its methylation might also be altered and whether methylation is affected by the substance (alcohol or nicotine) consumed or by dependency is still unclear. Nevertheless, *GDAP1* methylation holds potential as a useful biomarker for SUD.

Authors disclosure

All authors have nothing to disclose.

Authors contribution

N.F. and A.M. designed the study. S.R. and M.P. conducted the experiments and analysis. M.V. treated the animals. E.M. provided the knockout mice and contributed to the manuscript. A.M., S.R. and N.F. wrote the manuscript. All authors approved the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Marie-Pierre Manitz and Jennifer Plümper for their constant, fast, and supportive advice regarding the methods. The study was financially supported by a FORUM grant (F896-17) by the department of medicine of the Ruhr-University Bochum.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2020.112979.

References

Abrous, D.N., Adriani, W., Montaron, M.-F., Aurousseau, C., Rougon, G., Le Moal, M., Piazza, P.V., 2002. Nicotine self-administration impairs hippocampal plasticity. J. Neurosci. Off. J. Soc. Neurosci 22, 3656–3662 https://doi.org/20026324.

Agrawal, A., Balasubramanian, S., Smith, E.K., Madden, P.A.F., Bucholz, K.K., Heath, A.C., Lynskey, M.T., 2010. Peer substance involvement modifies genetic influences on regular substance involvement in young women. Addict. Abingdon Engl. 105, 1844–1853. https://doi.org/10.1111/j.1360-0443.2010.02993.x.

American Psychiatric Association, 2013. Diagnostic and statistical manual of mental disorders (DSM-5®). American Psychiatric Pub.

Bachman, J., 2013. Immunohistochemistry on freely floating fixed tissue sections. Methods Enzymol 533, 207–215. https://doi.org/10.1016/B978-0-12-420067-8.00013-1.

Barneo-Muñoz, M., Juárez, P., Civera-Tregón, A., Yndriago, L., Pla-Martin, D., Zenker, J., Cuevas-Martín, C., Estela, A., Sánchez-Aragó, M., Forteza-Vila, J., Cuezva, J.M., Chrast, R., Palau, F., 2015. Lack of GDAP1 induces neuronal calcium and mitochondrial defects in a knockout mouse model of charcot-marie-tooth neuropathy. PLoS Genet. 11, e1005115. https://doi.org/10.1371/journal.pgen.1005115.

Brody, A.L., Mandelkern, M.A., London, E.D., Olmstead, R.E., Farahi, J., Scheibal, D., Jou, J., Allen, V., Tiongson, E., Chefer, S.I., Koren, A.O., Mukhin, A.G., 2006. Cigarette smoking saturates brain alpha 4 beta 2 nicotinic acetylcholine receptors. Arch. Gen. Psychiatry 63, 907–915. https://doi.org/10.1001/archpsyc.63.8.907.

Brückmann, C., Di Santo, A., Karle, K.N., Batra, A., Nieratschker, V., 2016. Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. Epigenetics 11, 456–463. https://doi.org/10.1080/15592294.2016.1179411.

Cecil, C.A.M., Walton, E., Smith, R.G., Viding, E., McCrory, E.J., Relton, C.L., Suderman, M., Pingault, J.-B., McArdle, W., Gaunt, T.R., Mill, J., Barker, E.D., 2016. DNA methylation and substance-use risk: a prospective, genome-wide study spanning gestation to adolescence. Transl. Psychiatry 6, e976. https://doi.org/10.1038/tp.2016.247.

Holliday, E.D., Nucero, P., Kutlu, M.G., Oliver, C., Connelly, K.L., Gould, T.J., Unterwald, E.M., 2016. Long-term effects of chronic nicotine on emotional and cognitive behaviors and hippocampus cell morphology in mice: comparisons of adult and adolescent nicotine exposure. Eur. J. Neurosci. 44, 2818–2828. https://doi.org/10.1111/ejn.13398.

Huizink, A.C., Levälahti, E., Korhonen, T., Dick, D.M., Pulkkinen, L., Rose, R.J., Kaprio, J., 2010. Tobacco, cannabis, and other illicit drug use among Finnish adolescent twins: causal relationship or correlated liabilities? J. Stud. Alcohol Drugs 71, 5–14. https://doi.org/10.15288/jsad.2010.71.5.

Hunsaker, M.R., Lee, B., Kesner, R.P., 2008. Evaluating the temporal context of episodic memory: the role of CA3 and CA1. Behav. Brain Res. 188, 310–315. https://doi.org/10.1016/j.bbr.2007.11.015.

John, D., Shelukhina, I., Yanagawa, Y., Deuchars, J., Henderson, Z., 2015. Functional alpha7 nicotinic receptors are expressed on immature granule cells of the postnatal dentate gyrus. Brain Res. 1601, 15–30. https://doi.org/10.1016/j.brainres.2014.12.041.

Kaneko, N., Okano, H., Sawamoto, K., 2006. Role of the cholinergic system in regulating survival of newborn neurons in the adult mouse dentate gyrus and olfactory bulb. Genes Cells Devoted Mol. Cell. Mech. 11, 1145–1159. https://doi.org/10.1111/j.1365-2443.2006.01010.x.

Kempermann, G., Kuhn, H.G., Gage, F.H., 1997. Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc. Natl. Acad. Sci. U. S. A. 94, 10409–10414.

Kesner, R.P., 2007. A behavioral analysis of dentate gyrus function. Prog. Brain Res. 163, 567–576. https://doi.org/10.1016/S0079-6123(07)63030-1.

Koesling, D., Mergia, E., Russwurm, M., 2016. Physiological Functions of NO-Sensitive Guanylyl Cyclase Isoforms. Curr. Med. Chem. 23, 2653–2665. https://doi.org/10.2174/0929867323666160812145050.

Kutlu, M.G., Gould, T.J., 2016. Effects of drugs of abuse on hippocampal plasticity and hippocampus-dependent learning and memory: contributions to development and maintenance of addiction. Learn. Mem. 23, 515–533. https://doi.org/10.1101/lm.042192.116.

Mahna, D., Puri, S., Sharma, S., 2018. DNA methylation signatures: biomarkers of drug and alcohol abuse. Mutat. Res. 777, 19–28. https://doi.org/10.1016/j.mrrev.2018.06.002.

Meyerhoff, D.J., Tizabi, Y., Staley, J.K., Durazzo, T.C., Glass, J.M., Nixon, S.J., 2006. Smoking comorbidity in alcoholism: neurobiological and neurocognitive consequences. Alcohol. Clin. Exp. Res. 30, 253–264. https://doi.org/10.1111/j.1530-0277.2006.00034.x.

Niemann, A., Ruegg, M., La Padula, V., Schenone, A., Suter, U., 2005. Ganglioside-induced differentiation associated protein 1 is a regulator of the mitochondrial network: new implications for Charcot-Marie-Tooth disease. J. Cell Biol. 170, 1067–1078. https://doi.org/10.1083/jcb.200507087.

Pedrola, L., Espert, A., Valdés-Sánchez, T., Sánchez-Piris, M., Sirkowski, E.E., Scherer, S.S., Fariñas, I., Palau, F., 2008. Cell expression of GDAP1 in the nervous system and pathogenesis of Charcot-Marie-Tooth type 4A disease. J. Cell. Mol. Med. 12, 679–689. https://doi.org/10.1111/j.1582-4934.2007.00158.x.

Perry, D.C., Dávila-García, M.I., Stockmeier, C.A., Kellar, K.J., 1999. Increased nicotinic receptors in brains from smokers: membrane binding and autoradiography studies. J. Pharmacol. Exp. Ther. 289, 1545–1552.

Philibert, R.A., Gunter, T.D., Beach, S.R.H., Brody, G.H., Madan, A., 2008. MAOA methylation is associated with nicotine and alcohol dependence in women. Am. J. Med. Genet. Part B Neuropsychiatr. Genet. Off. Publ. Int. Soc. Psychiatr. Genet. 147B, 565–570. https://doi.org/10.1002/ajmg.b.30778.

Philibert, R.A., Penaluna, B., White, T., Shires, S., Gunter, T., Liesveld, J., Erwin, C., Hollenbeck, N., Osborn, T., 2014. A pilot examination of the genome-wide DNA methylation signatures of subjects entering and exiting short-term alcohol dependence treatment programs. Epigenetics 9, 1212–1219. https://doi.org/10.4161/epi.32252.

Phillips, B., Veljkovic, E., Peck, M.J., Buettner, A., Elamin, A., Guedj, E., Vuillaume, G., Ivanov, N.V., Martin, F., Boué, S., Schlage, W.K., Schneider, T., Titz, B., Talikka, M., Vanscheeuwijck, P., Hoeng, J., Peitsch, M.C., 2015. A 7-month cigarette smoke inhalation study in C57BL/6 mice demonstrates reduced lung inflammation and emphysema following smoking cessation or aerosol exposure from a prototypic modified risk tobacco product. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 80, 328–345. https://doi.org/10.1016/j.fct.2015.03.009.

Razin, A., Cedar, H., 1991. DNA methylation and gene expression. Microbiol. Rev. 55, 451–458.

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: imageJ for the next generation of scientific image data. BMC Bioinformatics 18, 529. https://doi.org/10.1186/s12859-017-1934-z.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Shen, J., Yakel, J.L., 2009. Nicotinic acetylcholine receptor-mediated calcium signaling in the nervous system. Acta Pharmacol. Sin. 30, 673–680. https://doi.org/10.1038/aps.2009.64.

Shingo, A.S., Kito, S., 2005. Effects of nicotine on neurogenesis and plasticity of hippocampal neurons. J. Neural Transm. Vienna Austria 1996 112, 1475–1478. https://doi.org/10.1007/s00702-005-0370-2.

Stone, A.L., Becker, L.G., Huber, A.M., Catalano, R.F., 2012. Review of risk and protective factors of substance use and problem use in emerging adulthood. Addict. Behav. 37, 747–775. https://doi.org/10.1016/j.addbeh.2012.02.014.

Szabo, S.I., Zelles, T., Lendvai, B., 2008. Intracellular Ca2+ dynamics of hippocampal interneurons following nicotinic acetylcholine receptor activation. Neurochem. Int. 52, 135–141. https://doi.org/10.1016/j.neuint.2007.05.012.

Tavakoli, H.R., Hull, M., Michael Okasinski, L.T., 2011. Review of current clinical biomarkers for the detection of alcohol dependence. Innov. Clin. Neurosci. 8, 26–33.

Tazir, M., Hamadouche, T., Nouioua, S., Mathis, S., Vallat, J.-.M., 2014. Hereditary motor and sensory neuropathies or Charcot-Marie-Tooth diseases: an update. J. Neurol. Sci. 347, 14–22. https://doi.org/10.1016/j.jns.2014.10.013.

Walter, M., Gerhard, U., Duersteler-MacFarland, K.M., Weijers, H.-.G., Boening, J., Wiesbeck, G.A., 2006. Social factors but not stress-coping styles predict relapse in detoxified alcoholics. Neuropsychobiology 54, 100–106. https://doi.org/10.1159/000096991.

Wu, J., Gao, M., Taylor, D.H., 2014. Neuronal nicotinic acetylcholine receptors are important targets for alcohol reward and dependence. Acta Pharmacol. Sin. 35, 311–315. https://doi.org/10.1038/aps.2013.181.

Zeid, D., Kutlu, M.G., Gould, T.J., 2018. Differential effects of nicotine exposure on the hippocampus across lifespan. Curr. Neuropharmacol. 16, 388–402. https://doi.org/10.2174/1570159X15666170714092436.

Zhang, H., Gelernter, J., 2016. DNA methylation and alcohol use disorders: progress and challenges. Am. J. Addict. https://doi.org/10.1111/ajad.12465.

Lithium and glutamine synthetase: Protective effects following stress

Annakarina Mundorf [a,b], Alexandra Knorr [a], Charlotte Mezö [b], Christina Klein [a], Dominik KE Beyer [a,b], Andreas J Fallgatter [b], Michael Schwarz [c], Nadja Freund [a,b]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, 44780 Bochum, Germany
[b] Clinic for Psychiatry and Psychotherapy, University of Tuebingen, Tuebingen, Germany
[c] Institute of Pharmacology and Toxicology, University of Tuebingen, Tuebingen, Germany

Mundorf, A., Knorr, A., Mezö, C., Klein, C., Beyer, D.K., Fallgatter, A.J., Schwarz, M., Freund, N (2019). Lithium and glutamine synthetase: Protective effects following stress. *Psychiatry Res* Aug;281:112544, https://doi.org/10.1016/j.psychres.2019.112544

Psychiatry Research 281 (2019) 112544

Contents lists available at ScienceDirect

Psychiatry Research

journal homepage: www.elsevier.com/locate/psychres

Lithium and glutamine synthetase: Protective effects following stress

Annakarina Mundorf[a,b], Alexandra Knorr[a], Charlotte Mezö[b], Christina Klein[a], Dominik KE Beyer[a,b], Andreas J Fallgatter[b], Michael Schwarz[c], Nadja Freund[a,b,*]

[a] Division of Experimental and Molecular Psychiatry, Department of Psychiatry, Psychotherapy and Preventive Medicine, LWL University Hospital, Ruhr-University Bochum, Bochum, 44780, Germany
[b] Clinic for Psychiatry and Psychotherapy, University of Tuebingen, Tuebingen, Germany
[c] Institute of Pharmacology and Toxicology, University of Tuebingen, Tuebingen, Germany

ARTICLE INFO

Keywords:
Reporter mouse model
Wnt/β-catenin signaling
Sex differences

ABSTRACT

Even though lithium is widely used as treatment for mood disorders, the exact mechanisms of lithium in the brain remain unknown. A potential mechanism affects the downstream target of the Wnt/β-catenin signaling pathway, specifically glutamine synthetase (GS). Here, we investigate the effect of lithium on GS-promoter activity in the brain. Over seven days, B6C3H-Glultm$^{(T2A-LacZ)}$ mice that carry LacZ as a reporter gene fused to the GS-promotor received either daily intraperitoneal injections of lithium carbonate (25 mg/kg) or NaCl, or no treatment. Following histochemical staining of β-galactosidase relative GS-promotor activity was measured by analyzing the intensity of the staining. Furthermore cell counts were conducted. GS-promotor activity was significantly decreased in female compared to male mice. Treatment group differences were only found in male hippocampi, with increased activity after NaCl treatment compared to both the lithium treatment and no treatment. Lithium treatment increased the overall number of cells in the CA1 region in males. Daily injections of NaCl might have been sufficient to induce stress-related GS-promotor activity changes in male mice; however, lithium was able to reverse the effect. Taken together, the current study indicates that lithium acts to prevent stress, rather affecting general GS-promoter activity.

GABA	Gamma-Aminobutyric acid
GS	Glutamine synthetase
GSK-3β	Glycogen synthase kinase 3β
MANOVA	Multivariate analysis of variance
NaCl	Natrium Chloride
X-gal	Beta-galactosidase

1. Introduction

The number of people diagnosed with mental disorders is increasing worldwide. Even in high-income countries, between 35% and 50% of people diagnosed with a mental illness go untreated (World Health Organization, 2018). These individuals, especially with mood disorders such as unipolar or bipolar depression, have a higher risk of attempting suicide (Tondo and Baldessarini, 2016). Multiple studies have shown that lithium treatment decreases the risk of suicide in patients with major mood disorders (Tondo and Baldessarini, 2016). The exact mechanism of action of lithium in the brain however, is still unclear.

The therapeutic range of lithium is narrow and there is a high risk of lithium intoxication during treatment. There are also various side effects of long-term lithium use including reduced urinary concentrating ability, hypothyroidism, hyperparathyroidism, weight gain, and with high doses, potential neurotoxicity (McKnight et al., 2012; Simard et al., 1989; Suraya and Yoong, 2001). Understanding the exact mechanisms of lithium in the brain is crucial for the development of medication targeting specific signaling pathways. This ultimately would reduce the side effects of lithium treatment. To date, the different mechanisms of action of lithium in the brain and the whole organism (Oruch et al., 2014) include influencing the gene regulation process (Lenox and Wang, 2003), modulating serotonin release in specific cells (Scheuch et al., 2010), and potentially altering the circadian rhythm by inhibiting the β-glycogen synthase kinase 3 (GSK-3β) (Yin et al., 2006). The inhibition of the GSK-3β might be the answer, as it is an enzyme that participates in the Wnt/β-catenin signaling pathway (Klein and Melton, 1996; Lenox and Wang, 2003; Oruch et al., 2014; Yin et al., 2006). The Wnt/β-catenin pathway is involved in various cellular processes including cell proliferation, cell development, cell survival and motility, as well as postnatal and adult brain plasticity and adult neurogenesis (Klein and Melton, 1996; Maguschak and Ressler, 2012). Alterations in this signaling pathway can cause changes

* Corresponding author.
E-mail address: nadja.freund@rub.de (N. Freund).

https://doi.org/10.1016/j.psychres.2019.112544
Received 26 February 2019; Received in revised form 29 August 2019; Accepted 29 August 2019
Available online 30 August 2019
0165-1781/

in behavior and cognition (Jin et al., 2017; Maguschak and Ressler, 2012). One downstream target of the Wnt/β-catenin signaling pathway is glutamine synthetase (GS) (Cadoret et al., 2002; Kordes et al., 2008). GS is crucial for the maintenance of the glutamate-glutamine-cycle and neurotransmission. GS recycles the neurotransmitter glutamate (Rose et al., 2013), as neurons are incapable of de novo synthesis of glutamate. Given the important role of glial GS in several metabolic pathways, such as the glutamate-glutamine-cycle and metabolism of ammonia (Rose et al., 2013), it is no surprise that alterations in GS expression are associated with several neurological disorders including depression, schizophrenia, epilepsy, Parkinson's, and Alzheimer's disease (Bruneau et al., 2005; Gruenbaum et al., 2015; Hensley et al., 1995; Kalkman, 2011; Kulijewicz-Nawrot et al., 2013; Rose et al., 2013; Yu et al., 2012).

In a study in rats, researchers found an increase of GS activity in the brain after 7 days of lithium treatment (Marcus et al., 1986). Such a finding is supported by the reduced GS activity found in the postmortem brains of depressed patients, as well as suicide cases of schizophrenic patients (Kalkman, 2011). Given the protective effect of lithium against suicide (Tondo and Baldessarini, 2016), and reduced GS activity in postmortem brains in suicide cases, the question arises whether lithium acts by elevating GS activity.

In the current study, we analyzed the effect of intraperitoneal 25 mg/kg lithium carbonate injections on GS-reporter activity in the brain of a GS-reporter mouse model. Reporter genes such as the LacZ gene can be fused to the promotor of a target gene due to transgenic techniques to facilitate the analysis of gene expression of a particular promoter. This model was used to determine the actual effect of lithium treatment on GS-promoter activity as a follow up to the findings by Marcus and colleagues (1986). Due to the fact that injection stress can affect the animal (Freund et al., 2013), a naïve control group with no prior treatment was included (no treatment group). Both male and female mice were analyzed in this study.

2. Material and methods

2.1. Animals

A GS-reporter mouse model, B6C3H-Glultm$^{(T2A\text{-}LacZ\text{-}loxP\text{-}T2A\text{-}Tk\text{-}1\text{-}FRT\text{-}loxP\text{-}T2A\text{-}Fluc\text{-}FRT)Arte}$, obtained from the Institute of Experimental and Clinical Pharmacology and Toxicology at the University of Tübingen, Germany (originally prepared by Taconic Artemis, Cologne, Germany) was chosen. Rapid changes in promoter activity can be detected using a reporter mouse. In this study, one out of the three reporters present in the transgene was utilized, namely the LacZ reporter, which allows quantification of its substrate beta-galactosidase. The LacZ reporter includes a nuclear localization signal in the LacZ open reading frame (SV40 monopartite nuclear localization signal PKKKRKV (cctaagaagaaggaaggaaggtt)) facilitating the quantification of beta-galactosidase-positive cells. Counting positives is more accurate as staining can easily be seen concentrated in the nucleus.

All animals were housed in a 12/12-hour dark-light cycle and under constant conditions (22 ± 2 °C). The animals had free access to food and water. For the first five generations (F1–F5), heterozygous GS-reporter mice were bred with C3H mice (Charles River, Germany). Generations F6 and F7 were bred within the heterozygous GS-reporter mice. For this study, only heterozygous GS-reporter mice of the F8 generation (F7 generation bred with C3H) were used. All procedures were approved by the local Animal Welfare and Ethics committee of the Country Commission Tübingen, Germany.

2.2. Drug treatment

Following the genotyping for the reporter gene, 23 three-month old female GS-reporter mice, and 19 three-month old male GS-reporter mice, were randomly assigned to one of three experimental groups. The first group was given daily intraperitoneal 25 mg/kg lithium carbonate in 0.1 ml 0.9% natrium chloride (NaCl) injections (100 mg Li_2CO_3 dissolved in 15 ml NaCl) for seven consecutive days (females $n = 8$, males $n = 6$). The chosen dose of 25 mg/kg results in a lithium blood level comparable to 0.8 meq/l in patients (Smithberg and Dixit, 1982). The second group received daily 0.1 ml NaCl injections (weight-independent) and served as an injection/stress control (females $n = 9$, males $n = 6$). The third group remained untreated and served as a no stress control (females $n = 6$, males $n = 7$).

2.3. Staining

Twenty-four hours after the last injection, all animals were anesthetized with ketamine hydrochloride and xylazine hydrochloride (100 mg/kg and 10 mg/kg, intraperitoneal). Transcranial perfusions were performed using 0.12 M sodium phosphate buffer (pH 7.4) followed by 4% paraformaldehyde, as a fixative. Perfusion surgeries were performed as previously described (Gage et al., 2012). Brains were coronally cryosectioned into 40 µm slices from rostral to caudal. To detect LacZ gene activity, beta-galactosidase (X-gal) staining was used. The staining protocol of Kokubu and Lim (2014) was followed, with the modifications outlined below. In brief, free-floating sections were incubated with 1 mg/ml X-gal staining solution for 3 h at 37 °C. The slices were washed and coverslipped with Mowiol.

For general cell counting in the hippocampus, Nissl staining was conducted with a different set of slices to stain DNA and RNA in the cell nucleus by cresyl violet. After X-gal staining, the slices were dehydrated in 70% ethanol and incubated with 1% cresyl violet (Sigma) solution for 3 min. Sections were differentiated in 96% ethanol mixed with a few drops of acetic acid. After dehydration, the slices were treated with isopropanol followed by xylene and coverslipped with DPX (Fluka).

Double staining of beta-galactosidase and GS-protein were also performed. Staining for GS-protein was performed according to standard free-floating immunohistochemistry staining protocols (Bachman, 2013). Slices were briefly incubated with the primary antibody anti-GS from rabbit by GenTex (1:500, GTX109121, overnight 4 °C) in 0.12 M PBS with 0.3% triton (Triton-X100, Sigma-Aldrich) and 1% normal goat serum (Sigma–Aldrich) and the secondary antibody anti-rabbit from goat (1:300, Sigma-Aldrich) for one hour. This was followed by incubation with an avidin-biotin-complex (Vector Laboratories, Vectastain ® ABC Kit) for one hour at room temperature in 1% A + 1% B in PBST. Final staining was by a peroxidase-catalyzed diaminobenzidine reaction (Vector Laboratories, DAB-Kit). Slices were washed in PBS and then stained with X-gal (see above).

2.4. Analysis

Thirteen coronal sections, each 40 µm in thickness between approximately 2.96 mm and −2.14 mm of bregma position, were investigated. The mouse brain atlas by Paxinos and Franklin (2001) was used to determine the level of sections across all animals. X-gal stained slices were imaged with the highest resolution (12.800 dpi) by using a transmitted light scanner (Epson Perfection V600 Photo). Images from single slices were transformed to gray scale, and were further analyzed with Matlab. The gray intensity of the background was first determined. Next the gray intensity of every pixel with a threshold higher than the background was measured. Finally, the mean gray intensity was calculated for each slide.

The hippocampi (at bregma ~ −1.92 mm), of the male animals were cropped with photoshop and converted into greyscale, before mean gray intensities were calculated. In the same layer of hippocampus (bregma ~ −1.92 mm), X-gal and cresyl violet stained cells in the CA1 and CA3 area were photographed with a transmitted light microscope (Olympus BX51) and the software CellD. Cells were counted manually by an investigator blinded to the terms of experimental conditions.

2.5. Statistics

Statistics were performed with SPSS 25. For analysis of gray intensities, Treatment (3: lithium vs NaCl vs no treatment) and Sex (2: male vs female) were set as factors to conduct a multivariate analysis of variance (MANOVA), with the different Brain Sections as variables. Student's *t*-test was applied for post-hoc comparison of Sex for all brain sections. Due to the main effect of Sex, the analysis was also conducted separately for male and female mice. After the MANOVA with Treatment (3: lithium vs NaCl vs no treatment) as the factor and Brain Sections as variables, a one-way ANOVA was conducted for each brain section separately. Gray intensities in the male hippocampus were compared with a one-way ANOVA using Treatment (3: lithium vs NaCl vs no treatment) as the factor. A MANOVA with Treatment as a factor (3: lithium vs NaCl vs no treatment) and Brain Region (CA1, CA3) as a variable was performed separately for each of the cell counts (X-gal positive cells and total cell number). A one-way ANOVA with Treatment as factor was followed for each region. Due to homogeneity in variance and a small difference in sample size, the Gabriel test was chosen as post-hoc test for treatment.

3. Results

3.1. Staining

The X-gal stained cells showed mostly nuclear staining of the GS-reporter. This results from a nuclear localization signal in the LacZ open reading frame in the GS promoter-driven transgene of the GS-reporter mouse. Double staining for X-gal and GS showed co-localization of staining for the GS-reporter and GS-protein, demonstrating the specificity of the GS-reporter (Fig. 1).

3.2. Lower GS-reporter activity in females

Using Pillai's trace, no significant differences between Treatment groups ($V = 0.68$; $F(26, 50) = 0.99$, $p = 0.49$) or an interaction between Treatment and Sex ($V = 0.76$; $F(26, 50) = 1.18$, $p = 0.29$) were evident, but a significant Sex difference ($V = 0.59$; $F(13, 24) = 2.714$, $p = 0.017$) was found. The measured relative gray intensity was significantly lower in females compared to males (Student's *t*-test $t(26) = -3.464$, $p = 0.002$) indicating less GS-reporter activity in females. When comparing the treatment groups separately, only the NaCl treatment group showed a significant difference (Student's *t*-test $t(13) = -4.207$, $p = 0.001$; not corrected for multiple testing, Fig. 2A).

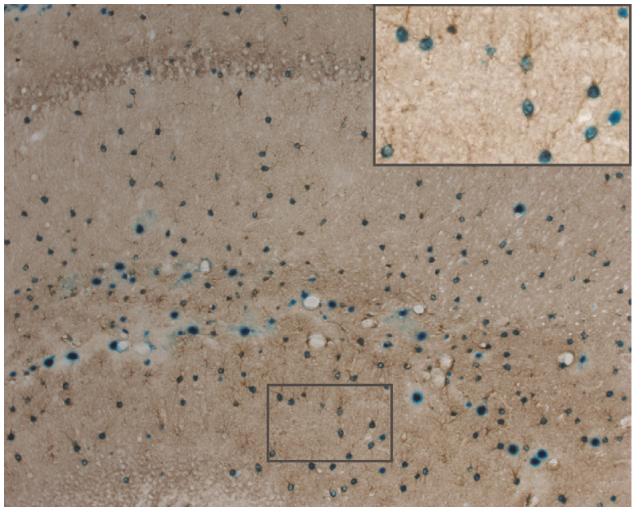


Fig. 1. Histochemically stained cells of a male mouse hippocampus. Beta-galactosidase representing the GS-reporter is stained blue, and glutamine synthetase protein is stained in brown. Nearly all cells with the reporter gene present also contained GS-protein and vice versa. Magnification in 20x and 40x for the right upper corner. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Treatment-Related changes in GS-reporter activity in male mice

Due to the main effect of Sex, we conducted statistical analysis separately for both male and female mice. No significant difference between groups were found in females ($V = 1.26$; $F(26, 18) = 1.18$, $p = 0.36$) and males ($V = 1.68$; $F(26, 10) = 2.08$, $p = 0.11$). However, when conducting the one-way ANOVA for each section level, section level 12 in males (at bregma ~ −1.92 mm) showed a significant effect of treatment ($F(2, 16) = 3.651$, $p = 0.049$; not corrected for multiple testing). The post-hoc test revealed a difference that approached significance between the NaCl and lithium groups (Gabriel, $p = 0.075$).

3.4. GS-reporter activity differences in the hippocampus

The one-way ANOVA showed a significant Treatment effect, $F(2, 16) = 4.466$, $p = 0.029$ in the hippocampus at bregma ~ −1.92 mm. The post-hoc Gabriel test revealed an increased gray intensity for the NaCl group compared to the lithium ($p = 0.051$) and no treatment groups ($p = 0.059$). The relative gray intensity did not differ between lithium and no treatment group ($p = 0.997$) (Fig. 2B).

3.5. Overall cell number in hippocampal region CA1 increased in lithium-treated males

After detecting effects in the hippocampus, X-gal stained cells and all Nissl stained cells were counted in discrete regions (Fig. 3A). The number of X-gal positive cells showed no significant differences for Treatment ($V = 0.17$; $F(4, 72) = 0.15$, $p = 0.96$) or Sex ($V = 0.12$; $F(2, 35) = 2.58$, $p = 0.09$) using Pillai's trace. The total cell numbers revealed differences in Sex ($V = 0.4$; $F(2, 35) = 11.86$, $p < 0.001$) and an interaction of Treatment and Sex ($V = 0.28$, $F(4, 72) = 3.02$, $p = 0.023$).

There was no main Treatment effect in males ($V = 0.44$, $F(4, 329) = 2.312$, $p = 0.079$), however there was an individual effect for the CA1 region ($F(2, 16) = 3.783$, $p = 0.045$; Fig. 3B). The Gabriel post-hoc analysis indicated differences between lithium-NaCl treatment ($p = 0.077$) and no treatment ($p = 0.087$) but not for NaCl treatment and no treatment ($p = 1.00$) in the CA1 region. No differences were detected in CA3 ($F(2, 16) = 0.199$, $p = 0.8$; Fig. 3B). In addition, there was no Treatment main effect in females ($V = 0.3$, $F(4, 40) = 1.81$, $p = 0.14$) nor was there an individual effect of region (CA1: $F(2, 20) = 0.742$, $p = 0.489$; CA3: $F(2, 20) = 1.743$, $p = 0.20$).

4. Discussion

GS is a key enzyme in neurotransmitter homeostasis in neuronal cells. The enzyme is relatively stable showing a long half-life (13–22 h) after synthesis (Suárez et al., 2002). To monitor rapid responses following exogenous stimuli, measurements of enzyme content or activity are of limited value only. We therefore investigated the activity of a GS-reporter in a transgenic mouse model, as it enabled us to detect rapid changes in the activation of the promoter-driven reporter following its activation. Double staining confirmed that GS-reporter activity was only detected in cells also expressing GS-protein (Fig. 1).

In this study, a significant effect of treatment on the GS-reporter activity in male mice was shown (Fig. 2B). In the hippocampus of males, GS-reporter activity increased in the NaCl group compared to the lithium and no treatment groups (Fig. 2B). Cell counting following X-gal and Nissl staining of cells in the hippocampus CA1 and CA3 regions (Fig. 3A), revealed significantly more Nissl stained cells in lithium-

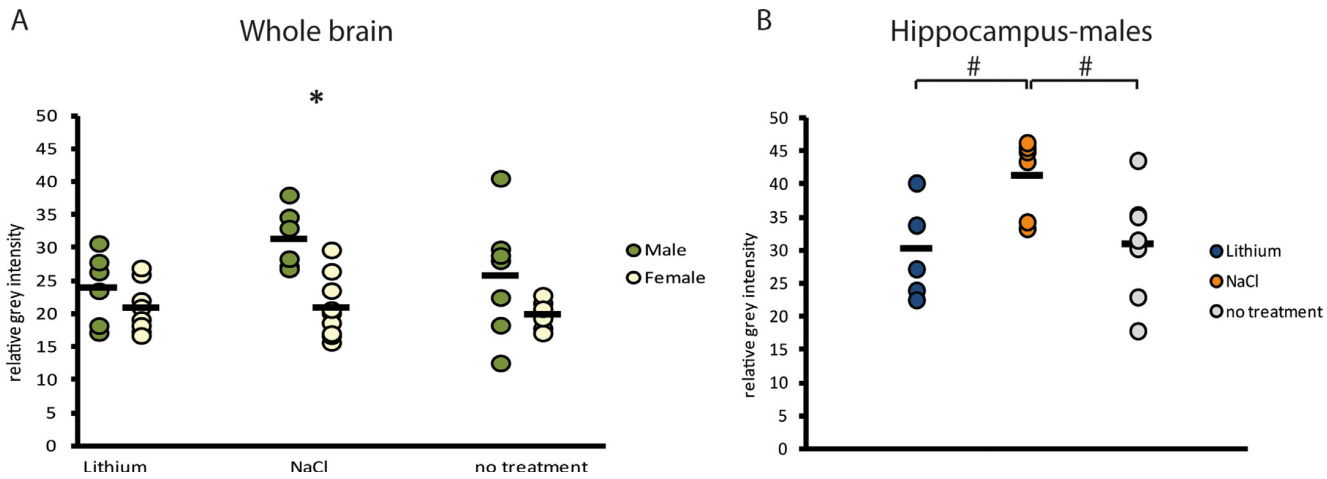


Fig. 2. GS-reporter activity. Data is presented as data points. The black bar represents the mean. A: Females specifically in the NaCl group show less GS-reporter activity (* $p < 0.05$). B: In the male hippocampus (at Bregma −1.92 mm) NaCl treatment resulted in increased activity of the GS-reporter (# $p < 0.06$).

treated males in the CA1 region, compared to NaCl and no treatment groups (Fig. 3B). There was no difference in total number of cells found in the CA3 region for all groups. In female mice, we found no differences in cell numbers between groups for both regions.

The increased GS-reporter activity found in the NaCl group compared to the lithium and no treatment groups was at first surprising. However, repeated daily intraperitoneal injections, even if only saline solution, must be regarded as a mild stressor (Deutsch-Feldman et al., 2015; Freund et al., 2013; Izumi et al., 1997), and have previously been shown to induce depressive-like behavior in rats (Izumi et al., 1997). Therefore, the increase of GS-reporter activity after daily NaCl injection could be the result of stress caused by the injections.

The influence of stress on the glutamate-glutamine-cycle, in which GS plays an important part, has previously been studied. Stress exposure in rodents led to increased prefrontal cortex glutamate (Popoli et al., 2011), and generally decreased gamma-aminobutyric acid (GABA) (synthesized from glutamate) levels in the brain (Skilbeck et al., 2010; Ulrich-Lai and Herman, 2009). Furthermore, hippocampal GABA levels increased when rats were given a stimulating stressor, and decreased when the animals were experiencing a possible negatively perceived stressor (de Groote and Linthorst, 2007). Given that the GS is part of the glutamate-glutamine-cycle and thereby influences the GABA deposit in synapses, it is likely that stress affects GS-expression. Our results show that an increase in GS-reporter activity in the NaCl treated male mice may indicate that the male mice experienced the daily injection as a negative stressor.

Interestingly, lithium-treated male mice significantly differed from the NaCl treated group, and showed GS-reporter activity levels comparable to the no treatment group. This finding indicates that lithium injections prevented an increase in GS-reporter activity as a stress response (as seen in the NaCl injected male mice). The results of the male mice indicate a protective role of lithium on the stress response as no differences in GS-reporter activity was found between lithium-treated and no treatment male mice, but a significantly increase in GS-reporter activity was seen in NaCl treated mice. Given the positive effect of lithium on cell proliferation and neuronal differentiation (Dong et al., 2015; Su et al., 2007; Zanni et al., 2015), a protective effect is likely. At first, this seems to contrast with the results of Marcus and colleges (1986) who reported an increase in the GS activity after lithium treatment. However, they investigated GS activity which might differ from the direct promoter activity we measured by the reporter activity. Moreover, Marcus and colleagues only observed an increase in activity in the brain stem (Marcus et al., 1986), which may not reflect changes in other brain regions.

In female mice, no difference in GS-reporter activity were found between all three treatment groups. This finding might indicate that female mice did not show a stress response to the injections. The fact, that male and female rodents respond differently to stress is already well known (Freund et al., 2013; Leussis et al., 2012). Male rats respond differently to a severe stressor, such as inescapable shocks, compared to females (Leussis et al., 2012). When given a mild stressor, such as NaCl injections, female but not male rats show beneficial effects (Freund et al., 2013). The beneficial effect of stress for female rats in contrast to male rats, has also been reported in a spatial memory task after restraint stress, regardless of the estrus (Bouma et al., 2009). Therefore, it is likely, that females did not react to the given injection procedure as did males in this experiment. The fact that a significant sex difference in GS-reporter activity was only seen in the NaCl treatment group (Fig. 2A), further demonstrates that only males may have reacted to the stressor. In this study, female mice were not categorized based upon their estrus cycle phase, therefore a potential hormonal effect on the stress response cannot be excluded.

A recently published study found significantly higher GS-protein levels in female than in male juvenile rats (Al-Suwailem et al., 2018). The study by Al-Suwailem and colleagues (2018) also reported lower levels of glutamate, as well as other factors, in the glutamate signaling pathway in females. The sex differences in the glutamate signaling pathway is assumed to have a neuroprotective effect in females compared to males, as high glutamate levels can have a neuroexcitotoxic effect (Al-Suwailem et al., 2018). This finding contrasts with our findings where we report less GS-reporter activity in female mice compared to males. However, measured GS-protein levels do not have to correspond to measured GS promoter activity measured by GS-reporter activity. Van Straaten and colleagues (2006) claimed a difference (up to 20-fold) in the murine GS-protein:mRNA ratio indicating a strong transcriptional and posttranscriptional regulation of the GS, whereas posttranscriptional regulation may not have a high impact (van Straaten et al., 2006). Moreover, higher GS levels in juvenile female rats found by Al-Suwailem and colleagues may not necessarily stabilize until adulthood. Given the increase of GS activity in juvenile female rats compared to male juvenile rats, it seems likely that brains prime differently during development based on sex. Furthermore, even though we did not see an interaction of sex and treatment, the main sex effect might be driven by the NaCl treatment group, and therefore reflect differences in stress response rather that a general increase in GS promoter activity in males.

The significantly higher number of cells in male mice CA1 region after lithium carbonate injection, in contrast to NaCl treatment or no

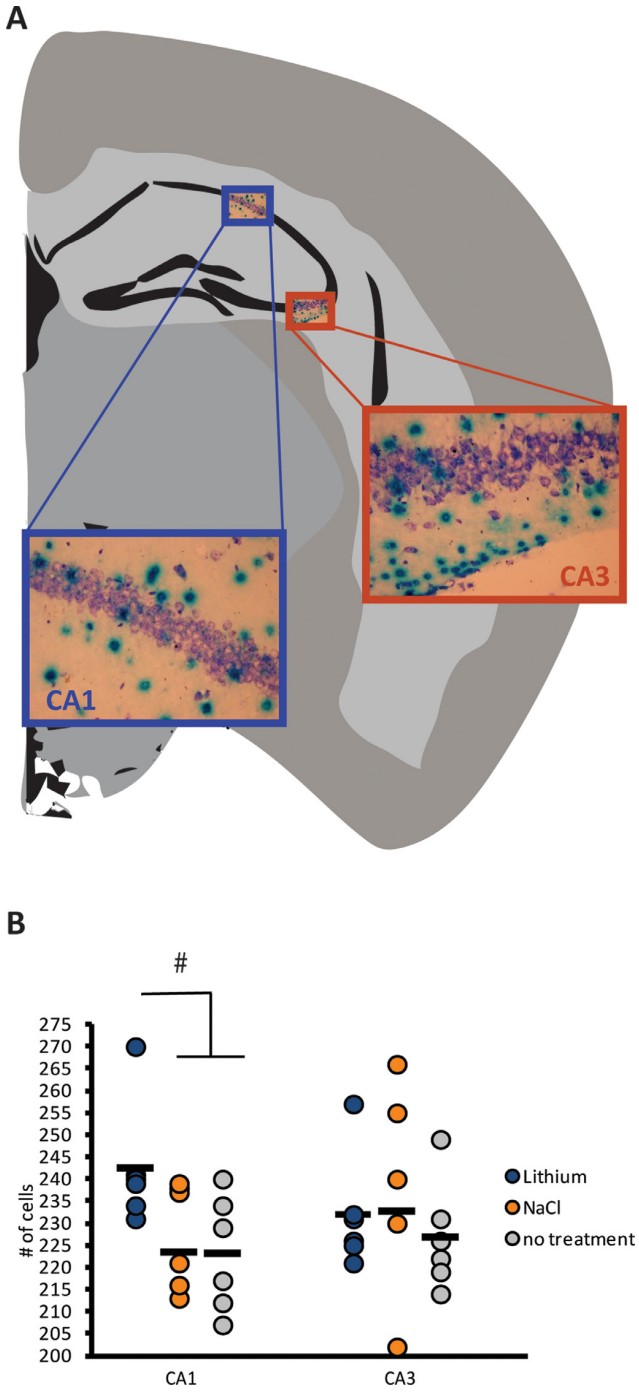


Fig. 3. Effect of treatment on cell numbers in male hippocampus. **A**: Nissl (violet) and GS-reporter positive cells (blue) were counted in the CA1 and the CA3 region of the hippocampus (bregma −1.92 mm). Nissl stained cells containing the GS-reporter appear predominantly blue as X-gal staining concealed Nissl staining; **B**: Total number of counted X-gal and Nissl stained cells in the CA1 and CA3. Total number of cells was increased in lithium-treated mice in CA1 (# $p < 0.1$). Data is presented as data points. The black bar represents the mean. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

treatment, is in line with other studies that identify an increase in CA1 cells after lithium treatment (Schaeffer et al., 2017). In a study examining the neuroprotective effects of lithium for hippocampal cell loss in a mouse model for Alzheimer's disease, male mice were treated with subtherapeutic and therapeutic doses of lithium at 3 months of age until 11 months of age, by lithium-supplemented chow (to exclude the stress of injection), where the subtherapeutic doses led to a significant increase of CA1 neurons in wild-type mice (Schaeffer et al., 2017).

The results of our study may indicate that lithium has a positive effect on cell proliferation in the CA1 region of male mice after 1 week with a therapeutic dose of lithium carbonate of 25 mg/kg per day. Therefore, it seems likely that to have positive effects on neuronal growth, the dose and length of lithium treatment depend on environmental influences such as stress. To our knowledge, studies on the effects of lithium on cell proliferation in the CA3 region are still rare. However, our study reports no difference in cell numbers in the CA3 region in all three treatment groups for both sexes. One possible explanation for increased cell proliferation in male CA1 but not in CA3 region could be the effect of stress. In a study from Watanabe and colleagues (1992), rats were restrained for 21 consecutive days. Afterwards, the researchers found a significant decrease of apical dendrites of CA3 neurons, but no change in CA1 or dentate gyrus neurons (Watanabe et al., 1992). If lithium acts as a protector against cell atrophy, this could be the reason why we found neither a decrease in cell numbers in the CA3 due to stress, nor an increase in cells compared to the CA1 region.

The not altered cell number in the CA3 after stress would be in line with the finding of Silva and colleagues (2008) who confirmed in a rat study the influence of lithium on both, neurogenesis and apoptosis in the hippocampus by regulating the activity of the GSK-3β. In their study, chronic-mild-stressed rats showed a decrease in hippocampal dentate cell turnover, however the decrease could be prevented with an accompanied lithium treatment. More interestingly, it seemed that control animals with lithium treatment showed an increase in cell proliferation and apoptosis in the hippocampus (Silva et al., 2008). These results confirm the controversial mechanisms of lithium found in therapeutic usage where lithium elevates depressive mood (Tondo and Baldessarini, 2016) while also attenuating a manic state (Beyer and Freund, 2017).

Given the protective effect of lithium treatment on cell turnover found in the rat hippocampus, lithium treatment to prevent suicide in patients might protect the brain against acute negative stress. In this case, GS may play a pivotal role in stress coping mechanisms of the brain. For future patient treatment, it would be interesting to investigate whether GS levels in blood or spinal fluid could predict the lithium dose needed for a protective effect. Moreover, it would be helpful to investigate whether stress assessment of patients before or during lithium treatment can be correlated to the lithium dose needed for a protective effect. Finally, it would be interesting to further investigate inflammatory pathways and activity of the GS-reporter, as GS plays a major role in regulating inflammatory processes in the brain (Palmieri et al., 2017). Lithium, on the other hand, might reduce inflammatory mediators and enzymes involved by inhibiting the GSK-3β signaling pathway (Nassar and Azab, 2014).

The results of our study indicate that lithium may act as a protective factor against stress by inhibiting an upregulation of GS expression particularly in the brain of male mice. Moreover, it seems that lithium might not only protect against stress, but also stimulate adult cell growth in males.

CRediT authorship contribution statement

Annakarina Mundorf: Data curation, Formal analysis, Writing - original draft. **Alexandra Knorr:** Data curation, Formal analysis. **Charlotte Mezö:** Data curation, Formal analysis. **Christina Klein:** Data curation, Formal analysis. **Dominik KE Beyer:** Data curation, Formal analysis. **Andreas J Fallgatter:** Conceptualization. **Michael Schwarz:** Conceptualization, Data curation, Formal analysis. **Nadja Freund:** Conceptualization, Writing - original draft.

Declaration of Competing Interest

The authors declare no conflict of interest.

Acknowledgments

We would like to thank Lucas B. Rizzo for conducting preliminary experiments, and Marie-Pierre Manitz for her constant, fast, and supportive advice regarding the methods. We would also like to thank Britta S. Thompson and Melissa Johnston for proof reading the manuscript. Moreover, we would like to thank the fortüne – Forschungsförderung der Tübinger Medizinischen Fakultät for funding (to NF).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.112544.

References

Al-Suwailem, E., Abdi, S., El-Ansary, A., 2018. Sex differences in the glutamate signaling pathway in juvenile rats. J. Neurosci. Res 96, 459–466. https://doi.org/10.1002/jnr.24144.

Bachman, J., 2013. Immunohistochemistry on freely floating fixed tissue sections. Methods Enzymol. 533, 207–215. https://doi.org/10.1016/B978-0-12-420067-8.00013-1.

Beyer, D.K.E., Freund, N., 2017. Animal models for bipolar disorder: from bedside to the cage. Int. J. Bipolar Disord. 5, 35. https://doi.org/10.1186/s40345-017-0104-6.

Bouma, E.M.C., Riese, H., Ormel, J., Verhulst, F.C., Oldehinkel, A.J., 2009. Adolescents' cortisol responses to awakening and social stress; effects of gender, menstrual phase and oral contraceptives. The TRAILS study. Psychoneuroendocrinology 34, 884–893. https://doi.org/10.1016/j.psyneuen.2009.01.003.

Bruneau, E.G., McCullumsmith, R.E., Haroutunian, V., Davis, K.L., Meador-Woodruff, J.H., 2005. Increased expression of glutaminase and glutamine synthetase mRNA in the thalamus in schizophrenia. Schizophr. Res. 75, 27–34. https://doi.org/10.1016/j.schres.2004.12.012.

Cadoret, A., Ovejero, C., Terris, B., Souil, E., Lévy, L., Lamers, W.H., Kitajewski, J., Kahn, A., Perret, C., 2002. New targets of beta-catenin signaling in the liver are involved in the glutamine metabolism. Oncogene 21, 8293–8301. https://doi.org/10.1038/sj.onc.1206118.

de Groote, L., Linthorst, A.C.E., 2007. Exposure to novelty and forced swimming evoke stressor-dependent changes in extracellular GABA in the rat hippocampus. Neuroscience 148, 794–805. https://doi.org/10.1016/j.neuroscience.2007.06.030.

Deutsch-Feldman, M., Picetti, R., Seip-Cammack, K., Zhou, Y., Kreek, M.J., 2015. Effects of handling and vehicle injections on adrenocorticotropic and corticosterone concentrations in Sprague-Dawley compared with Lewis rats. J. Am. Assoc. Lab. Anim. Sci. JAALAS 54, 35–39.

Dong, B.-T., Tu, G.-J., Han, Y.-X., Chen, Y., 2015. Lithium enhanced cell proliferation and differentiation of mesenchymal stem cells to neural cells in rat spinal cord. Int. J. Clin. Exp. Pathol. 8, 2473–2483.

Freund, N., Thompson, B.S., Denormandie, J., Vaccarro, K., Andersen, S.L., 2013. Windows of vulnerability: maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. Neuroscience 249, 88–97. https://doi.org/10.1016/j.neuroscience.2013.06.066.

Gage, G.J., Kipke, D.R., Shain, W., 2012. Whole animal perfusion fixation for rodents. J. Vis. Exp. JoVE (65), 3564. https://doi.org/10.3791/3564.

Gruenbaum, S.E., Wang, H., Zaveri, H.P., Tang, A.B., Lee, T.-S.W., Eid, T., Dhaher, R., 2015. Inhibition of glutamine synthetase in the central nucleus of the amygdala induces anhedonic behavior and recurrent seizures in a rat model of mesial temporal lobe epilepsy. Epilepsy Behav. 51, 96–103. https://doi.org/10.1016/j.yebeh.2015.07.015.

Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., Aksenova, M., Gabbita, S.P., Wu, J.F., Carney, J.M., 1995. Brain regional correspondence between Alzheimer's disease histopathology and biomarkers of protein oxidation. J. Neurochem. 65, 2146–2156.

Izumi, J., Washizuka, M., Hayashi-Kuwabara, Y., Yoshinaga, K., Tanaka, Y., Ikeda, Y., Kiuchi, Y., Oguchi, K., 1997. Evidence for a depressive-like state induced by repeated saline injections in Fischer 344 rats. Pharmacol. Biochem. Behav. 57, 883–888.

Jin, X., Li, T., Zhang, L., Ma, J., Yu, L., Li, C., Niu, L., 2017. Environmental enrichment improves spatial learning and memory in vascular dementia rats with activation of Wnt/β-catenin signal pathway. Med. Sci. Monit. Int. Med. J. Exp. Clin. Res. 23, 207–215. https://doi.org/10.12659/MSM.902728.

Kalkman, H.O., 2011. Circumstantial evidence for a role of glutamine-synthetase in suicide. Med. Hypotheses 76, 905–907. https://doi.org/10.1016/j.mehy.2011.03.005.

Klein, P.S., Melton, D.A., 1996. A molecular mechanism for the effect of lithium on development. Proc. Natl. Acad. Sci. U S A 93, 8455–8459.

Kokubu, H., Lim, J., 2014. X-gal staining on adult mouse brain sections. Bio-Protoc 4.

Kordes, C., Sawitza, I., Häussinger, D., 2008. Canonical Wnt signaling maintains the quiescent stage of hepatic stellate cells. Biochem. Biophys. Res. Commun. 367, 116–123. https://doi.org/10.1016/j.bbrc.2007.12.085.

Kulijewicz-Nawrot, M., Syková, E., Chvátal, A., Verkhratsky, A., Rodríguez, J.J., 2013. Astrocytes and glutamate homoeostasis in Alzheimer's disease: a decrease in glutamine synthetase, but not in glutamate transporter-1, in the prefrontal cortex. ASN Neuro. 5, 273–282. https://doi.org/10.1042/AN20130017.

Lenox, R.H., Wang, L., 2003. Molecular basis of lithium action: integration of lithium-responsive signaling and gene expression networks. Mol. Psychiatry 8, 135–144. https://doi.org/10.1038/sj.mp.4001306.

Leussis, M.P., Freund, N., Brenhouse, H.C., Thompson, B.S., Andersen, S.L., 2012. Depressive-like behavior in adolescents after maternal separation: sex differences, controllability, and GABA. Dev. Neurosci. 34, 210–217. https://doi.org/10.1159/000339162.

Maguschak, K.A., Ressler, K.J., 2012. A role for WNT/β-Catenin signaling in the neural mechanisms of behavior. J. Neuroimmune. Pharmacol. Off. J. Soc. NeuroImmune Pharmacol. 7, 763–773. https://doi.org/10.1007/s11481-012-9350-7.

Marcus, S.R., Nadiger, H.A., Chandrakala, M.V., Rao, T.I., Sadasivudu, B., 1986. Acute and short-term effects of lithium on glutamate metabolism in rat brain. Biochem. Pharmacol. 35, 365–369.

McKnight, R.F., Adida, M., Budge, K., Stockton, S., Goodwin, G.M., Geddes, J.R., 2012. Lithium toxicity profile: a systematic review and meta-analysis. Lancet Lond. Engl. 379, 721–728. https://doi.org/10.1016/S0140-6736(11)61516-X.

Nassar, A., Azab, A.N., 2014. Effects of lithium on inflammation. ACS Chem. Neurosci. 5, 451–458. https://doi.org/10.1021/cn500038f.

Oruch, R., Elderbi, M.A., Khattab, H.A., Pryme, I.F., Lund, A., 2014. Lithium: a review of pharmacology, clinical uses, and toxicity. Eur. J. Pharmacol. 740, 464–473. https://doi.org/10.1016/j.ejphar.2014.06.042.

Palmieri, E.M., Menga, A., Lebrun, A., Hooper, D.C., Butterfield, D.A., Mazzone, M., Castegna, A., 2017. Blockade of glutamine synthetase enhances inflammatory response in microglial cells. Antioxid. Redox Signal. 26, 351–363. https://doi.org/10.1089/ars.2016.6715.

Paxinos, G., Franklin, K.B.J., 2001. The Mouse Brain Atlas in Stereotaxic Coordinates. Academic Press, USA.

Popoli, M., Yan, Z., McEwen, B.S., Sanacora, G., 2011. The stressed synapse: the impact of stress and glucocorticoids on glutamate transmission. Nat. Rev. Neurosci. 13, 22–37. https://doi.org/10.1038/nrn3138.

Rose, C.F., Verkhratsky, A., Parpura, V., 2013. Astrocyte glutamine synthetase: pivotal in health and disease. Biochem. Soc. Trans. 41, 1518–1524. https://doi.org/10.1042/BST20130237.

Schaeffer, E.L., Catanozi, S., West, M.J., Gattaz, W.F., 2017. Stereological investigation of the CA1 pyramidal cell layer in untreated and lithium-treated 3xTg-AD and wild-type mice. Ann. Anat. Anat. Anz. Off. Organ Anat. Ges. 209, 51–60. https://doi.org/10.1016/j.aanat.2016.10.002.

Scheuch, K., Höltje, M., Budde, H., Lautenschlager, M., Heinz, A., Ahnert-Hilger, G., Priller, J., 2010. Lithium modulates tryptophan hydroxylase 2 gene expression and serotonin release in primary cultures of serotonergic raphe neurons. Brain Res. 1307, 14–21. https://doi.org/10.1016/j.brainres.2009.10.027.

Silva, R., Mesquita, A.R., Bessa, J., Sousa, J.C., Sotiropoulos, I., Leão, P., Almeida, O.F.X., Sousa, N., 2008. Lithium blocks stress-induced changes in depressive-like behavior and hippocampal cell fate: the role of glycogen-synthase-kinase-3beta. Neuroscience 152, 656–669. https://doi.org/10.1016/j.neuroscience.2007.12.026.

Simard, M., Gumbiner, B., Lee, A., Lewis, H., Norman, D., 1989. Lithium carbonate intoxication. A case report and review of the literature. Arch. Intern. Med. 149, 36–46.

Skilbeck, K.J., Johnston, G.A.R., Hinton, T., 2010. Stress and GABA receptors. J. Neurochem. 112, 1115–1130. https://doi.org/10.1111/j.1471-4159.2009.06539.x.

Smithberg, M., Dixit, P.K., 1982. Teratogenic effects of lithium in mice. Teratology 26, 239–246. https://doi.org/10.1002/tera.1420260304.

Su, H., Chu, T.-H., Wu, W., 2007. Lithium enhances proliferation and neuronal differentiation of neural progenitor cells in vitro and after transplantation into the adult rat spinal cord. Exp. Neurol. 206, 296–307. https://doi.org/10.1016/j.expneurol.2007.05.018.

Suárez, I., Bodega, G., Fernández, B., 2002. Glutamine synthetase in brain: effect of ammonia. Neurochem. Int. 41, 123–142.

Suraya, Y., Yoong, K.Y., 2001. Lithium neurotoxicity. Med. J. Malays. 56, 378–381.

Tondo, L., Baldessarini, R.J., 2016. Suicidal behavior in mood disorders: response to pharmacological treatment. Curr. Psychiatry Rep. 18, 88. https://doi.org/10.1007/s11920-016-0715-0.

Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. 10, 397–409. https://doi.org/10.1038/nrn2647.

van Straaten, H.W.M., He, Y., van Duist, M.M., Labruyère, W.T., Vermeulen, J.L.M., van Dijk, P.J., Ruijter, J.M., Lamers, W.H., Hakvoort, T.B.M., 2006. Cellular concentrations of glutamine synthetase in murine organs. Biochem. Cell Biol. Biochim. Biol. Cell. 84, 215–231. https://doi.org/10.1139/o05-170.

Watanabe, Y., Gould, E., McEwen, B.S., 1992. Stress induces atrophy of apical dendrites of hippocampal CA3 pyramidal neurons. Brain Res. 588, 341–345.

World Health Organization, 2018. WHO | mental disorders [WWW document]. WHO. urlhttp://www.who.int/mediacentre/factsheets/fs396/en/(accessed 1.11.18).

Yin, L., Wang, J., Klein, P.S., Lazar, M.A., 2006. Nuclear receptor Rev-erbalpha is a critical lithium-sensitive component of the circadian clock. Science 311, 1002–1005. https://doi.org/10.1126/science.1121613.

Yu, X., He, G.-R., Sun, L., Lan, X., Shi, L.-L., Xuan, Z.-H., Du, G.-H., 2012. Assessment of the treatment effect of baicalein on a model of Parkinsonian tremor and elucidation of the mechanism. Life Sci. 91, 5–13. https://doi.org/10.1016/j.lfs.2012.05.005.

Zanni, G., Di Martino, E., Omelyanenko, A., Andäng, M., Delle, U., Elmroth, K., Blomgren, K., 2015. Lithium increases proliferation of hippocampal neural stem/progenitor cells and rescues irradiation-induced cell cycle arrest in vitro. Oncotarget 6, 37083–37097. https://doi.org/10.18632/oncotarget.5191.

Chapter 6 |
General discussion

The thesis aimed to disentangle epigenetic processes in terms of DNA methylation and gene expression in the context of brain development and psychopathology. Therefore, childhood and postpartum time have been identified as sensitive periods in rats resulting in stress-induced disruptions. In terms of neuronal structures, intra- and interhemispheric white matter alterations, the medial prefrontal cortex, and the hippocampus were repeatedly identified as target regions of both exposure and psychopathology. Two potential candidate genes were characterized regarding their sensitivity towards a specific exposure and psychopathology. Then, neuronal correlates of altered DNA methylation and gene expression of these interactions were analyzed allowing for deeper insights into the potential psychiatric application of DNA methylation.

6.1. Summary of key findings

The two translational review articles described in chapters 2 and 3 allow concluding that, given ELS as a risk factor for depression, certain induced changes after ELS are similar to the ones seen in depression. However, some alterations on the molecular level differ between ELS-exposed and non-exposed patients suggesting more than one mechanism being causative to psychopathology. The white matter volume steadily increases throughout development, reflecting the increasing number in connections and circuitries being formed and strengthened. Comprehensive findings of white matter alterations in depression indicate an important role of intact white matter integrity, especially as alterations were most pronounced in inter- and intrahemispheric connections. Investigating turning behavior after ELS supports the hypothesis of altered interhemispheric communication after ELS resulting in atypical laterality. Moreover, the comprehensive literature review in chapter 2 not only highlights the important role of white matter structures but also elucidates how changes on the molecular level lead to severe impairment in cognitive function. The studies on rats described in chapters 3 and 4 identified childhood and postpartum time as sensitive periods for stress-induced impairments. Interestingly, the induced phenotype differs in directionality throughout development emphasizing adolescence as a great time for particularly pronounced phenotype manifestation. Moreover, in both chapters, two factors mediating between exposure and phenotype are proposed: group-housing and maternal care. In chapter 5, two potential candidate genes,

MORC1 and *GDAP1*, were characterized regarding their sensitivity towards a specific exposure (stress, cigarette smoke) and psychopathology (depression, substance use disorder). Then, neuronal correlates of altered DNA methylation and gene expression of these interactions were analyzed allowing for deeper insights into a potential psychiatric application of DNA methylation. Lastly, the two projects on genetically modified mouse models revealed that target gene expression is dynamic as a response to exposure even when exposure is experienced in adulthood. Then, regions of ongoing neuronal growth such as the hippocampus are especially affected. Moreover, lithium treatment seems to protect against stress-induced alterations in hippocampal gene expression.

The studies presented in this thesis all highlight the important function of epigenetic influences during sensitive periods of brain development and their potential to induce long-lasting neurobiological consequences causative for psychopathology. However, some questions still arise concerning certain factors determining the severity of consequences of exposure on psychopathology that must be resolved, before further unraveling the role of epigenetic processes in the development of psychopathology.

6.2. Developmental trajectories of exposure

One aim of this thesis was to identify sensitive periods of stress exposure leading to long-lasting neurobiological alterations. The review article presented in chapter 2 indicates that alterations in white matter differ between depressive individuals having experienced childhood abuse and depressive patients without childhood exposure. Childhood as a critical time to induce long-lasting neurobiological changes is further highlighted in the comprehensive literature review included in chapter 3. On the behavioral level, ELS exposure resulted in altered anxiety and atypical laterality in animals (chapter 3) lining up with the results previously reported. Induced changes in neuronal gene expression become most relevant when these changes occur in regions maturing during time of exposure, therefore, regions of interest should be chosen depending on time of exposure. However, periods of early brain maturation might not be uniquely periods susceptible to impairments, as seen in chapter 4, indicating that the postpartum period is a time of great change as well. Given the *Morc1* gene expression analysis after MS in chapter 3 yielding no effect of exposure on mRNA levels, the need to disentangle the exact time in early life when neuronal *Morc1* expression is most vulnerable to alterations is highlighted. For certain genes (e.g., *Otx2*), the time of stress exposure being causative for altered gene expression is restricted to a narrowly defined period (Peña et al., 2017). Exposing e.g., mice to MS over differently selected postnatal periods, a distinct vulnerable period emerged when exposure

alters expression of certain genes and makes mice susceptible to second stress exposure in adulthood. This early priming then manifests in depression-like behavior only after the second exposure in adulthood (Peña et al., 2017). Interestingly, when analyzing gene expression patterns after exposure, different genes are affected after early compared to late-life exposure with only a small overlap between the two types. These vulnerable windows of development are also reflected in behavioral studies revealing different sensitive times for males and females (Freund et al., 2013). Consequently, discovering the exact time point when the expression of certain genes is most vulnerable to result in long-lasting harmful impairments is critical to deduce relevant epigenetic processes.

When disentangling the phenomenon of behavioral implications of ELS becoming most pronounced in adolescence, developmental trajectories are especially important to consider. Therewith, induced neuronal changes become more noticeable (by altered behavior) when neuronal circuits are being strengthened suggesting that early behavior might be more instinctive (e.g. juvenile rats being anxious when something is frightening) and thus regional impairment of brain function might not impact behavior. During development, when circuits are being formed and reinforced, the behavior might become more reasoned and influenced by experiences and emotions (e.g. adolescent rats become anxious when the environment is unknown or seemingly frightening). In adult mice, the emotional state is depending on innate or learned values as well and thus is influenced by experience or circumstances (e.g. a thirsty mouse has more pleasure when drinking) (Dolensek et al., 2020). Thus adolescent behavior might be more affected through their emotional state. The studies included in this thesis also implicate a reversing effect of exposure after group housing suggesting a dynamic pattern underlining susceptibility. Developmental symptom trajectories have also been studied in humans by analyzing longitudinal population-based data. These studies report subclinical externalizing or internalizing symptoms in children that develop into symptoms matching diagnostic criteria in adolescence (Cecil et al., 2014; Walton et al., 2017). It is proposed that ELS leads to accelerated developmental trajectories whereas current stress in adolescence results in delayed brain development in adolescence. This is reflected in a stronger maturational decrease in gray matter volume after ELS and a smaller decrease when experiencing current stress suggesting that ELS induces increased neuronal pruning during puberty (Tyborowska et al., 2018). Interestingly, the changes in gray matter volume are independently related to exposure meaning that experiencing both stressors does not result in a cumulative effect (Tyborowska et al., 2018).

Adolescent communication differs from juveniles and adults, independently of exposure as shown in chapter 3. In a test for anxiety, adolescent rats emitted significantly fewer ultrasonic calls compared to juveniles and adults without showing altered anxiety highlighting adolescence as a special time in general (Casey et al., 2008). Puberty is also the time with the greatest sex dimorphisms in brain growth with more sexually mature adolescents showing the greatest sex differences (Bramen et al., 2011). Thus, consequences of exposure are not only depending on age but also on the individual's level of sexual maturation, especially as ELS can accelerate sexual maturation in girls potentially via increase cortisol release (Mendle et al., 2011). Interestingly, MS in dams reduced *Morc1* expression in the medial prefrontal cortex suggesting postpartum as a time of neuronal vulnerability. As described in the comprehensive review article concerning alterations found in postpartum depression and MS dams (chapter 4), neuronal alterations after childbirth have been reported in women suffering from postpartum depression with hormonal changes being predominantly responsible for alterations (Brummelte & Galea, 2016; Deligiannidis et al., 2013; Xie et al., 2018). Given the critical time of puberty with enormous hormonal changes (Sisk & Foster, 2004) and the sensitive postpartum time also marked by great changes in hormone levels (Schiller et al., 2015), hormones affecting reproductive behavior are likely to be involved in forming neuronal vulnerability and resilience to exposure.

Considering brain plasticity, the hippocampus still remains able of neurogenesis in adulthood enabling ongoing neuronal plasticity and learning (Shors et al., 2012). The studies included in chapter 5 investigating consequences of exposure on hippocampal cells in adult mice revealed reduced *GDAP1* gene expression after exposure. Therefore, gene expression patterns are still sensitive to exposure in adulthood indicating that time of exposure is most relevant in terms of neuronal structure implicated, meaning stress in adulthood most likely will most likely affect hippocampal neurogenesis. Also, hippocampal neurons born in adulthood morphologically differ from neonatally-born neurons and show a prolonged development (Cole et al., 2020) rendering them more vulnerable for maturational impairments.

Developmental trajectories of brain development are thus important to consider when investigating the consequences of early exposure on neurobiology and behavior. The timing of exposure is relevant in determining which neuronal structure will be disrupted in normal development as well as relevant for the type of symptoms that will develop. Furthermore, hormonal levels might play an important role in the susceptibility of brain regions and should be considered as cofactors more frequently. As brain development is an ongoing process throughout life with some regions experiencing prolonged maturation, these regions are most

vulnerable to display disruptions. However, some alterations only become obvious in phenotypic changes after repeated exposure suggesting that early exposure primes the brain towards a susceptibility but does not necessarily lead to psychopathology. The studies included in chapter 3 confirmed the previously reported sensitive time of exposure but highlight the more critical time of adolescence. Induced changes in gene expression rendering the individual susceptible to further exposure are time-sensitive and thus, windows of vulnerability can be missed. Moreover, the studies included in this thesis implicate that the effects of exposure were reversed after group housing suggesting a dynamic pattern underlining susceptibility.

6.3. Implications of repeated stress exposure: Is there a need for a second hit?

Throughout this thesis, the question reoccurs whether repeated exposure by two time-independent stressors implicates a critical point towards resulting psychopathology. Studies yield different results supporting both, a cumulative effect of exposure as well as independently induced mechanisms leading to psychopathology. Studies included in chapter 3 suggest that early exposure, depending on time and duration, leads to different degrees of psychopathology, e.g., repeated exposure (MS followed by ISO) resulted in the greatest behavioral alterations in terms of atypical leftward laterality. However, in terms of anxiety, cumulative exposure did not lead to increased anxiety compared to MS or ISO alone, therefore, raising the question of whether a second hit is needed for symptom manifestation or not.

Generally, early stress exposure can change the sensitivity towards the pathogenic effects of stress throughout life, rendering the individual more vulnerable or resilient towards subsequent exposure (Kendler et al., 2004). Thus, the two-hit model proposes that early adverse life events (first hit) make the brain vulnerable whereas a stressor later in life (second hit) is triggering the onset of psychopathologies (Mc Elroy & Hevey, 2014; Worlein, 2014). Cumulative stress exposure is considered a high-risk factor for psychiatric disorders (de Kloet et al., 2005). Moreover, it is hypothesized, that every new stress exposure or psychiatric episode activates neurotoxic pathways resulting in a decline of brain structures along with the progress of the disorder (Belleau et al., 2019). Consequently, repeated stress exposure should result in a more severe decline in brain structures and increased psychopathology. This coincides with the study presented in chapter 3 on atypical laterality after ELS in rats, where repeated exposure (MS followed by ISO) resulted in the greatest behavioral alterations in terms of atypical leftward laterality. Thus, the two hit model holds great potential to elucidate the pathogenic effects of repeated stress exposure. However, the two hit model might be contingent on certain

circumstances, for example, early exposure renders mice susceptible to stress only when the exposure happened at a certain time. Then, in adults, early exposure manifests in depression-like behavior solely after a second exposure (Peña et al., 2017).

Opposing to the diathesis model is the match/mismatch hypothesis proposing that cumulative stress leads to an adaptive response rendering the individual less vulnerable or more adaptive to further stress whereas a mismatch between the early and later experience results in impairments (Daskalakis et al., 2012; Santarelli et al., 2014). This hypothesis is in line with studies included in the comprehensive review in chapter 2 revealing different implications after ELS than in depression without ELS, presumed that ELS serves as the first hit. Others report a cumulative effect of exposure only manifesting in certain behavioral changes whereas other phenotypes are most pronounced after a single exposure (Jaric et al., 2019). According to the match/mismatch theory, the cumulative exposure of rats in chapter 3 should have resulted in less anxiety and less atypical laterality compared to controls, or MS or ISO alone. However, as both were not the case it appears as if other factors influence the resulting susceptibility. Therefore, another point relevant for determining the effects of exposure is the emotional perception of the experienced stress or the emotional state while exposed to a stressor (Childs et al., 2014; Lazarus, 1974). Accordingly, a situation that is negatively perceived elicits a stronger negative reaction when reexperienced (second stressor) (Childs et al., 2014). As rats exposed to MS and ISO experience two consecutive stressors, their emotional state might be more aversive compared to only MS or IS rats, resulting in greater changes. It has already been revealed that in mice, emotions depend on inherited or learned values and thus are altered by experience or external conditions (Dolensek et al., 2020). An equally important factor in determining the susceptibility towards stress is the environment surrounding exposure. High levels of maternal behavior, for example, can buffer the effects of ELS as well as low levels of maternal care renders pups more susceptible to develop a maladaptive stress response (Weaver et al., 2004). More interestingly, maternal behavior therewith primes the individuals' stress reaction by altering hippocampal GC receptor gene methylation in the first postnatal week (D. Liu et al., 1997; Weaver et al., 2004). This methylation pattern can persist but can also be reversed in adulthood (Weaver et al., 2006). Given the contradicting results and the high influence of environmental factors, an integrated approach arises stating that the degree of early neuronal programming effects to stress susceptibility determines whether individuals are susceptible to a second stressor or not. For individuals experiencing strong programming effects of early exposure, the match/mismatch hypothesis will hold whereas the two hit or cumulative

stress approach is applicable for individuals encountering no or few programming consequences from early exposure (Nederhof & Schmidt, 2012).

There is a strong indication of a need for a second hit to trigger the onset of psychopathology. However, multiple factors are involved in shaping the susceptibility towards stress such as a certain time of exposure, the emotional perception of stressor, and mediating factors increasing or mitigating the effects evoked by the first exposure. In studies including human subjects, early adversity is predominantly assessed retrospectively and thus, not always accurate making it difficult to resolve the issue of a second stressor and programming effects influencing susceptibility. Given the strong impact of environmental factors, programming effects are likely to be regulated by epigenetic influences. To investigate the potential of DNA methylation patterns as a predictor of susceptibility, longitudinal studies are needed to enable the analysis of multiple time points throughout development (Cecil et al., 2014; 2020; Rijlaarsdam et al., 2017).

6.4. Potential applications of DNA methylation in psychiatry

Another aim of this thesis was to investigate whether the study of DNA methylation holds prospect as a biomarker for disorders and if altered methylation further reflects changes in gene expression causative for psychopathology. Studies included in chapter 5 indicate that methylation patterns of specific genes are suitable to serve as a marker of disease. Moreover, the first study in chapter 5 highlights the potential use of DNA methylation for the early detection of subclinical symptoms before they reach diagnostic criteria (Mundorf et al., 2018).

Even though the field of methylation as a biomarker reveals promising results, there are still some difficulties concerning a precise application. One difficulty is to identify disorder-specific genes, and thus corresponding methylation pattern. As outlined in the comprehensive summary article in chapter 3, certain genes are affected in various disorders (Matosin et al., 2017; Mundorf & Freund, 2019; Vinkers et al., 2015) rendering it difficult to distinguish between disorders based on genetic markers. However, in different psychiatric disorders, the same genes, and environmental risk factors are implicated (Argentieri et al., 2017; Palma-Gudiel & Fañanás, 2017), a high transition between disorders exist (Costello et al., 2003) and co-occurrence of different disorders (Bittner et al., 2007; Copeland et al., 2013). This points to a general dimension of psychopathology (Allegrini et al., 2020; Caspi et al., 2014; Selzam et al., 2018). This general dimension is proposed to directly influence all symptoms and reflects a general risk to develop any or all forms of psychopathologies, independent of the disorder (Caspi et al.,

2014). Therefore, studies should also focus on general markers for increased risk or categories of disorders grouped in symptom or neuronal deficit classes.

Another obstacle is that patterns of gene methylation are affected by substances e.g., cigarette smoke with such exposure resulting in altered hippocampal gene expression independent of psychopathology (Mundorf et al., 2020). Therefore, it remains difficult to distinguish if the changes found in methylation patterns are a result of consumption or other confounders e.g., addiction (C. Liu et al., 2018; Nielsen et al., 2012). Similar to substance use, methylation patterns can be influenced by pharmacological treatment (Boks et al., 2012; Ovenden et al., 2018). The last study in chapter 5 indicates that treatment with lithium, as a wildly used mood stabilizer, induces rapid changes in gene expression, acting therewith as a protective factor against stress-induced changes (Mundorf et al., 2019). Interestingly, psychotherapy can alter methylation patterns as well by aligning the pattern to one of healthy controls (Roberts et al., 2015, 2019). Besides the aligning effect of psychotherapy on methylation, the degree of change is predictive for treatment success with greater change in methylation pattern associated with a greater treatment response (Roberts et al., 2019) suggesting a new application of epigenetics in predicting treatment response. Future applications might also hold new treatment options for psychiatric disorders by targeting gene methylation directly. Subjecting animals to e.g., a methyl donor added to the diet improved anxiety and depressive-like behavior induced by preceded early exposure (McCoy et al., 2016; Paternain et al., 2016) inspiring the study of new approaches in humans as well. In the emerging field of epigenetic therapy, two classes of drugs are already being investigated: DNA methyltransferase and histone deacetylase inhibitors (Karsli-Ceppioglu, 2016; Kular & Kular, 2018; Peedicayil, 2012). Interestingly, psychotropic drugs already used in clinical practice, demonstrate epigenetic effects additive to the commonly known mechanisms of action (Ptak & Petronis, 2008). The mood stabilizer valproate, for example, also inhibits histone deacetylase (Ptak & Petronis, 2008). However, induced epigenetic alterations are not yet the main target of action of psychopharmacology as they demonstrate a lack of target specificity, and thus, their application would not only result in alterations of epimutations but also attract implications on other epigenetic patterns (Kular & Kular, 2018). More studies on agents targeting specific enzymes are needed to substantially increase target specificity first.

Studies conducted with human subjects usually investigate peripheral tissue but given that DNA methylation is tissue-specific, gene methylation in blood or buccal cells may not always be reflective of brain cell methylation patterns (Bakulski et al., 2016). Moreover, gene methylation patterns in peripheral cells can differ as well e.g., between blood and buccal cells

(Thomas et al., 2018) emphasizing the need to include different tissues to identify the one being most sensitive to reflect changes and thus making it suitable as a biomarker of disease. Besides, as psychiatric disorders are disorders of the brain, the question arises whether changes in peripheral tissue reflect changes in neuronal tissue and thus, might also be predictive of neuronal implications. In an exploratory study included in chapter 5, the association between peripheral *MORC1* methylation and neuronal density in the hippocampus and medial prefrontal cortex was investigated in humans but, even though a significant correlation between increased methylation and reduced neuronal density was found, the study does not allow to conclude that altered *MORC1* methylation is causative for neuronal impairments.

The study of DNA methylation patterns in psychiatry holds great potential for future clinical applications as a biomarker of disease facilitating early diagnostic and for predicting treatment response. Furthermore, epigenetic processes emerge as promising new targets for innovative pharmacotherapy. However, research in this field is still at the beginning and needs to face some challenges first. Although animal models enable overcome some obstacles, more focus should be placed on translational study design. For example, although animal models allow for the analysis of neuronal tissue, they often do not include peripheral tissue as well. Conveniently, for this specific challenge, new databases are rising to enable the analysis of blood-brain concordance of methylation patterns.

6.5. Conclusion and outlook

Epigenetic processes hold great prospects in understanding neuronal mechanisms implicated in psychiatric disorders, therewith enabling to define periods and mechanisms associated with increased susceptibility towards psychopathology and consequently, allowing for detection possibilities of early intervenience. However, as stated in the general discussion, some challenges arise in this innovative field of psychiatric epigenetics that research needs to conquer first.

One challenge can be overcome using longitudinal data enabling to analyze DNA methylation patterns at different times and thus, before and after exposure. However, only a few longitudinal, population-based studies of humans exists such as the Avon Longitudinal Study of Parents and Children (ALSPAC; Fraser et al., 2013) from England, the Dunedin study from New Zealand (Poulton et al., 2015) or the Generation R study from the Netherlands (Jaddoe et al., 2007; Kooijman et al., 2016). These prospective epidemiological studies monitor children and their parents gathering psychological and physiological information e.g. whole-genome

methylation data at different times and therefore enable the investigation of early environmental and genetic causes of normal and abnormal growth, development, and health from fetal life until young adulthood. Conducting studies within these datasets will enable analyzing multiple time points throughout development and thus, to investigate the potential of DNA methylation patterns as a predictor of susceptibility and to disentangle how epigenetic programming is regulated by environmental factors (Cecil et al., 2014; 2020; Rijlaarsdam et al., 2017). Moreover, these population-based studies mentioned are largely aligned facilitating to test the robustness of findings by running a replication analysis in one of the other data sets. Utilizing these data sets will enable future research to rise above the difficulty of retrospectively assessed early adversity. Furthermore, it will enable to separately analyze implications of early-exposed and non-exposed affected individuals. Given that these epidemiological studies monitor children and their parents, they hold comprehensive information concerning environmental factors influencing susceptibility further enabling insight into both, the two hits and the match/mismatch hypothesis.

Besides multiple time points of testing, studies on adversity, DNA methylation, and mental health mostly focus on candidate genes. This can present a problem as the candidate gene approach does not allow for the discovery of new genes, is often biased and thus, has a higher risk of false positives. Additionally, this approach only provides a limited scope as the development of most psychiatric disorders is likely to involve a broader set of genes. Consequently, the most promising approach to investigate a potential epigenetic mechanism has been shown to be epigenome-wide association studies (EWAS) (Barker et al., 2018). EWAS allows for the investigation of genome-wide DNA methylation patterns therewith enabling the identification of common normal variations in the DNA methylome (Flanagan, 2015). Concerning the implications of altered gene methylation as a cause or consequence of disorder, studies on candidate gene methylation often successfully report alterations in clinical samples. However, they fail to replicate their findings in pre-clinical samples indicating that the presented change in gene methylation is rather a biomarker of disease, but not causative for disease (Flanagan, 2015). Therefore, the most powerful way to identify causative markers of disease is to investigate common epigenetic variation (by EWAS) in the general population (Flanagan, 2015). However, as EWAS investigate genome-wide DNA methylation patterns the results present methylation patterns of approximately 450k different CpG sites, consequently entailing a higher statistical false positive rate and multiple testing errors. But again, researchers have risen to the challenge and are starting to create DNA methylation modules based upon the data derived from EWAS (Koopman-Verhoeff et al., 2020). These modules are identified and

clustered using a weighted gene co-expression network analysis revealing clusters of methylated CpG sites that are highly correlated and then, enable the analysis of DNA methylation networks comprised of genes that are potentially also functionally related (Botía et al., 2017).

Research needs to further tackle the question of whether altered DNA methylation is a marker of disease or causative for neuronal alterations such as reduced volume or neurite density as proposed in chapter 5. The studies investigating DNA methylation as a cause rather than a consequence of disorder are rare in humans but hold great potential for a more sophisticated approach. One reason for the lack of studies on neuronal consequences is the fact that most studies use peripheral tissue like blood or buccal cells to extract the DNA. But given that DNA methylation is tissue-specific, gene methylation in blood or buccal cells may not always be reflective of brain cell methylation patterns (Bakulski et al., 2016). Therefore, it should be of great relevance to investigate whether changes in peripheral DNA also reflect neuronal changes, and thus if studies including peripheral tissue also allow for the analysis of whether altered methylation is a cause of the disorder. Animal models hold great potential to overcome this obstacle as they enable the study of neuronal and peripheral tissue and enable control of certain environmental factors. Additionally, new methods are emerging that employ more sophisticated, statistical approaches paving the way for investigating the blood-brain concordance of methylation patterns using searchable databases that have compared genome-wide methylation patterns between peripheral and postmortem brain tissues (Braun, Han, Hing, et al., 2019; Hannon et al., 2015). One of these web-based search tools is IMAGE-CpG (Iowa Methylation Array Graphing for Experimental Comparison of Peripheral tissue & Gray matter). IMAGE-CpG reports Spearman's rho correlation coefficients between peripheral and gray matter tissue (which has been surgically removed in epilepsy patients) for the MethylationEPIC array (Braun, Han, Hing, et al., 2019; Braun, Han, Nagahama, et al., 2019). To resolve ongoing discussions about whether peripheral tissue methylation patterns can be equated with brain alterations, more studies should include these tools examining the blood-brain concordance of gene methylation.

Even though psychiatric epigenetics is facing some challenges now, the rapidly developing field of psychiatric epigenetics increasingly produces sophisticated tools and approaches in conquering the presented challenges certainly enabling to disentangle epigenetic influences involved in brain development and susceptibility towards psychopathology. Moreover, using approaches as the analysis of genome-wide DNA methylation networks in longitudinal data

sets and examining the blood-brain concordance of gene methylation will facilitate answering whether altered DNA methylation arises as a cause or consequence of the disorder.

References

Aisa, B., Tordera, R., Lasheras, B., Del Río, J., & Ramírez, M. J. (2008). Effects of maternal separation on hypothalamic-pituitary-adrenal responses, cognition and vulnerability to stress in adult female rats. *Neuroscience*, *154*(4), 1218–1226. https://doi.org/10.1016/j.neuroscience.2008.05.011

Alegría-Torres, J. A., Baccarelli, A., & Bollati, V. (2011). Epigenetics and lifestyle. *Epigenomics*, *3*(3), 267–277. https://doi.org/10.2217/epi.11.22

Alves, R. L., Portugal, C. C., Summavielle, T., Barbosa, F., & Magalhães, A. (2019). Maternal separation effects on mother rodents' behaviour: A systematic review. *Neuroscience and Biobehavioral Reviews*. https://doi.org/10.1016/j.neubiorev.2019.09.008

American Psychiatric Association. (2013). *Diagnostic and Statistical Manual DSM 5*. https://doi.org/10.1176/appi.books.9780890425596.744053

Andersen, S. L., & Teicher, M. H. (2004). Delayed effects of early stress on hippocampal development. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology*, *29*(11), 1988–1993. https://doi.org/10.1038/sj.npp.1300528

Andersen, S. L., & Teicher, M. H. (2008). Stress, sensitive periods and maturational events in adolescent depression. *Trends in Neurosciences*, *31*(4), 183–191. https://doi.org/10.1016/j.tins.2008.01.004

Andreasen, N. C. (2010). Posttraumatic stress disorder: A history and a critique. *Annals of the New York Academy of Sciences*, *1208*, 67–71. https://doi.org/10.1111/j.1749-6632.2010.05699.x

Anisman, H., Zaharia, M. D., Meaney, M. J., & Merali, Z. (1998). Do early-life events permanently alter behavioral and hormonal responses to stressors? *International Journal of Developmental Neuroscience*, *16*(3–4), 149–164. https://doi.org/10.1016/S0736-5748(98)00025-2

Aragam, N., Wang, K.-S., & Pan, Y. (2011). Genome-wide association analysis of gender differences in major depressive disorder in the Netherlands NESDA and NTR population-based samples. *Journal of Affective Disorders*, *133*(3), 516–521. https://doi.org/10.1016/j.jad.2011.04.054

Argentieri, M. A., Nagarajan, S., Seddighzadeh, B., Baccarelli, A. A., & Shields, A. E. (2017). Epigenetic Pathways in Human Disease: The Impact of DNA Methylation on Stress-Related Pathogenesis and Current Challenges in Biomarker Development. *EBioMedicine*, *18*, 327–350. https://doi.org/10.1016/j.ebiom.2017.03.044

Bakulski, K. M., Halladay, A., Hu, V. W., Mill, J., & Fallin, M. D. (2016). Epigenetic Research in Neuropsychiatric Disorders: The "Tissue Issue." *Current Behavioral Neuroscience Reports*, *3*(3), 264–274. https://doi.org/10.1007/s40473-016-0083-4

Barker, E. D., Roberts, S., & Walton, E. (2018). Hidden hypotheses in "hypothesis-free" genome-wide epigenetic associations. *Current Opinion in Psychology*, *27*, 13–17. https://doi.org/10.1016/j.copsyc.2018.07.009

Barker, E. D., Walton, E., & Cecil, C. A. M. (2017). Annual Research Review: DNA methylation as a mediator in the association between risk exposure and child and adolescent psychopathology. *Journal of Child Psychology and Psychiatry, and Allied Disciplines*. https://doi.org/10.1111/jcpp.12782

Beach, S. R. H., Brody, G. H., Barton, A. W., & Philibert, R. A. (2016). Exploring genetic moderators and epigenetic mediators of contextual and family effects: From Gene × Environment to epigenetics. *Development and Psychopathology*, 1–14. https://doi.org/10.1017/S0954579416000882

Beattie, R., & Hippenmeyer, S. (2017). Mechanisms of radial glia progenitor cell lineage progression. *FEBS Letters*, *591*(24), 3993–4008. https://doi.org/10.1002/1873-3468.12906

Becker, M., Weinberger, T., Chandy, A., & Schmukler, S. (2016). Depression During Pregnancy and Postpartum. *Current Psychiatry Reports*, *18*(3), 1–9. https://doi.org/10.1007/s11920-016-0664-7

Beery, A. K., McEwen, L. M., MacIsaac, J. L., Francis, D. D., & Kobor, M. S. (2016). Natural variation in maternal care and cross-tissue patterns of oxytocin receptor gene methylation in rats. *Hormones and Behavior*, *77*, 42–52. https://doi.org/10.1016/j.yhbeh.2015.05.022

Behnia, F., Parets, S. E., Kechichian, T., Yin, H., Dutta, E. H., Saade, G. R., Smith, A. K., & Menon, R. (2015). Fetal DNA methylation of autism spectrum disorders candidate genes: Association with spontaneous preterm birth. *American Journal of Obstetrics and Gynecology*, *212*(4), 533.e1-9. https://doi.org/10.1016/j.ajog.2015.02.011

Belleau, E. L., Treadway, M. T., & Pizzagalli, D. A. (2019). The Impact of Stress and Major Depressive Disorder on Hippocampal and Medial Prefrontal Cortex Morphology. *Biological Psychiatry*, *85*(6), 443–453. https://doi.org/10.1016/j.biopsych.2018.09.031

Bilbo, S. D., & Schwarz, J. M. (2012). The immune system and developmental programming of brain and behavior. *Frontiers in Neuroendocrinology*, *33*(3), 267–286. https://doi.org/10.1016/j.yfrne.2012.08.006

Billiards, S. S., Haynes, R. L., Folkerth, R. D., Trachtenberg, F. L., Liu, L. G., Volpe, J. J., & Kinney, H. C. (2006). Development of microglia in the cerebral white matter of the human fetus and infant. *The Journal of Comparative Neurology*, *497*(2), 199–208. https://doi.org/10.1002/cne.20991

Blakemore, S.-J. (2012). Development of the social brain in adolescence. *Journal of the Royal Society of Medicine*, *105*(3), 111–116. https://doi.org/10.1258/jrsm.2011.110221

Boccia, M. L., Razzoli, M., Vadlamudi, S. P., Trumbull, W., Caleffie, C., & Pedersen, C. A. (2007). Repeated Long Separations from Pups Produces Depression-like Behavior in Rat Mothers. *Psychoneuroendocrinology*, *32*(1), 65–71. https://doi.org/10.1016/j.psyneuen.2006.10.004

Boks, M. P., de Jong, N. M., Kas, M. J. H., Vinkers, C. H., Fernandes, C., Kahn, R. S., Mill, J., & Ophoff, R. A. (2012). Current status and future prospects for epigenetic psychopharmacology. *Epigenetics*, *7*(1), 20–28. https://doi.org/10.4161/epi.7.1.18688

Botía, J. A., Vandrovcova, J., Forabosco, P., Guelfi, S., D'Sa, K., United Kingdom Brain Expression Consortium, Hardy, J., Lewis, C. M., Ryten, M., & Weale, M. E. (2017). An additional k-means clustering step improves the biological features of WGCNA gene co-expression networks. *BMC Systems Biology*, *11*(1), 47. https://doi.org/10.1186/s12918-017-0420-6

Bousalham, R., Benazzouz, B., Hessni, A. E., Ouichou, A., & Mesfioui, A. (2013). Maternal Separation Affects Mothers ' Affective and Reproductive Behaviors as Well as Second Offspring ' s Emotionality. *Test*, *2013*(September), 409–414. http://dx.doi.org/10.4236/jbbs.2013.35042

Brain Development Cooperative Group. (2012). Total and regional brain volumes in a population-based normative sample from 4 to 18 years: The NIH MRI Study of Normal Brain Development. *Cerebral Cortex (New York, N.Y.: 1991)*, *22*(1), 1–12. https://doi.org/10.1093/cercor/bhr018

Bramen, J. E., Hranilovich, J. A., Dahl, R. E., Forbes, E. E., Chen, J., Toga, A. W., Dinov, I. D., Worthman, C. M., & Sowell, E. R. (2011). Puberty influences medial temporal lobe and cortical gray matter maturation differently in boys than girls matched for sexual maturity. *Cerebral Cortex (New York, N.Y.: 1991)*, *21*(3), 636–646. https://doi.org/10.1093/cercor/bhq137

Braun, P. R., Han, S., Hing, B., Nagahama, Y., Gaul, L. N., Heinzman, J. T., Grossbach, A. J., Close, L., Dlouhy, B. J., Howard, M. A., Kawasaki, H., Potash, J. B., & Shinozaki, G. (2019). Genome-wide DNA methylation comparison between live human brain and peripheral tissues within individuals. *Translational Psychiatry*, *9*(1), 1–10. https://doi.org/10.1038/s41398-019-0376-y

Braun, P. R., Han, S., Nagahama, Y., Gaul, L., Heinzman, J., Hing, B., Grossbach, A., Dlouhy, B., Howard, M., Kawasaki, H., Potash, J., & Shinozaki, G. (2019). 28 - IMAGE-CpG: DEVELOPMENT OF A WEB-BASED SEARCH TOOL FOR GENOME-WIDE DNA METHYLATION CORRELATION BETWEEN LIVE HUMAN BRAIN AND PERIPHERAL TISSUES WITHIN INDIVIDUALS. *European Neuropsychopharmacology*, *29*, S796. https://doi.org/10.1016/j.euroneuro.2017.08.029

Brenhouse, H. C., Lukkes, J. L., & Andersen, S. L. (2013). Early life adversity alters the developmental profiles of addiction-related prefrontal cortex circuitry. *Brain Sciences*, *3*(1), 143–158. https://doi.org/10.3390/brainsci3010143

Brückmann, C., Di Santo, A., Karle, K. N., Batra, A., & Nieratschker, V. (2016). Validation of differential GDAP1 DNA methylation in alcohol dependence and its potential function as a biomarker for disease severity and therapy outcome. *Epigenetics*, *11*(6), 456–463. https://doi.org/10.1080/15592294.2016.1179411

Brummelte, S., & Galea, L. A. M. (2010). Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. *Hormones and Behavior*, *58*(5), 769–779. https://doi.org/10.1016/j.yhbeh.2010.07.012

Brummelte, S., & Galea, L. A. M. (2016). Postpartum depression: Etiology, treatment and consequences for maternal care. *Hormones and Behavior*, *77*, 153–166. https://doi.org/10.1016/j.yhbeh.2015.08.008

Burmeister, M., McInnis, M. G., & Zöllner, S. (2008). Psychiatric genetics: Progress amid controversy. *Nature Reviews Genetics*, *9*(7), 527–540. https://doi.org/10.1038/nrg2381

Burns, A., & Iliffe, S. (2009). Alzheimer's disease. *BMJ (Clinical Research Ed.)*, *338*, b158. https://doi.org/10.1136/bmj.b158

Carr, C. P., Martins, C. M. S., Stingel, A. M., Lemgruber, V. B., & Juruena, M. F. (2013). The role of early life stress in adult psychiatric disorders: A systematic review according to childhood trauma subtypes. *The Journal of Nervous and Mental Disease*, *201*(12), 1007–1020. https://doi.org/10.1097/NMD.0000000000000049

Casey, B. J., Getz, S., & Galvan, A. (2008). The adolescent brain. *Developmental Review: DR*, *28*(1), 62–77. https://doi.org/10.1016/j.dr.2007.08.003

Cecil, C A M, Lysenko, L. J., Jaffee, S. R., Pingault, J.-B., Smith, R. G., Relton, C. L., Woodward, G., McArdle, W., Mill, J., & Barker, E. D. (2014). Environmental risk, Oxytocin Receptor Gene (OXTR) methylation and youth callous-unemotional traits: A 13-year longitudinal study. *Molecular Psychiatry*, *19*(10), 1071–1077. https://doi.org/10.1038/mp.2014.95

Cecil, Charlotte A. M., Zhang, Y., & Nolte, T. (2020). Childhood maltreatment and DNA methylation: A systematic review. *Neuroscience and Biobehavioral Reviews*, *112*, 392–409. https://doi.org/10.1016/j.neubiorev.2020.02.019

Champagne, D. L., Ronald de Kloet, E., & Joëls, M. (2009). Fundamental aspects of the impact of glucocorticoids on the (immature) brain. *Seminars in Fetal and Neonatal Medicine*, *14*(3), 136–142. https://doi.org/10.1016/j.siny.2008.11.006

Chaudhury, S., Sharma, V., Kumar, V., Nag, T. C., & Wadhwa, S. (2016). Activity-dependent synaptic plasticity modulates the critical phase of brain development. *Brain & Development*, *38*(4), 355–363. https://doi.org/10.1016/j.braindev.2015.10.008

Chen, J., Evans, A. N., Liu, Y., Honda, M., Saavedra, J. M., & Aguilera, G. (2012). Maternal deprivation in rats is associated with corticotropin releasing hormone (CRH) promoter hypomethylation and enhances CRH transcriptional responses to stress in adulthood. *Journal of Neuroendocrinology*, *24*(7), 1055–1064. https://doi.org/10.1111/j.1365-2826.2012.02306.x

Childs, E., White, T. L., & de Wit, H. (2014). Personality traits modulate emotional and physiological responses to stress. *Behavioural Pharmacology*, *25*(5 0 6), 493–502. https://doi.org/10.1097/FBP.0000000000000064

Cole, J. D., Espinueva, D., Seib, D. R., Ash, A. M., Cooke, M. B., Cahill, S. P., O'Leary, T., Kwan, S. S., & Snyder, J. S. (2020). Adult-born hippocampal neurons undergo extended

development and are morphologically distinct from neonatally-born neurons Prolonged development of adult-born neurons. *Journal of Neuroscience*. https://doi.org/10.1523/JNEUROSCI.1665-19.2020

Costello, E. J., Mustillo, S., Erkanli, A., Keeler, G., & Angold, A. (2003). Prevalence and development of psychiatric disorders in childhood and adolescence. *Archives of General Psychiatry*, *60*(8), 837–844. https://doi.org/10.1001/archpsyc.60.8.837

Dantzer, R., & Kelley, K. W. (2007). Twenty years of research on cytokine-induced sickness behavior. *Brain, Behavior, and Immunity*, *21*(2), 153–160. https://doi.org/10.1016/j.bbi.2006.09.006

Daskalakis, N. P., Oitzl, M. S., Schächinger, H., Champagne, D. L., & de Kloet, E. R. (2012). Testing the cumulative stress and mismatch hypotheses of psychopathology in a rat model of early-life adversity. *Physiology & Behavior*, *106*(5), 707–721. https://doi.org/10.1016/j.physbeh.2012.01.015

de Kloet, E. R., Joëls, M., & Holsboer, F. (2005). Stress and the brain: From adaptation to disease. *Nature Reviews Neuroscience*, *6*(6), 463–475. https://doi.org/10.1038/nrn1683

Deligiannidis, K. M., Sikoglu, E. M., Shaffer, S. A., Frederick, B., Svenson, A. E., Kopoyan, A., Kosma, C. A., Rothschild, A. J., & Moore, C. M. (2013). GABAergic neuroactive steroids and resting-state functional connectivity in postpartum depression: A preliminary study. *Journal of Psychiatric Research*, *47*(6), 816–828. https://doi.org/10.1016/j.jpsychires.2013.02.010

Dennis, K. E., & Levitt, P. (2005). Regional expression of brain derived neurotrophic factor (BDNF) is correlated with dynamic patterns of promoter methylation in the developing mouse forebrain. *Molecular Brain Research*, *140*(1), 1–9. https://doi.org/10.1016/j.molbrainres.2005.06.014

Dolensek, N., Gehrlach, D. A., Klein, A. S., & Gogolla, N. (2020). Facial expressions of emotion states and their neuronal correlates in mice. *Science (New York, N.Y.)*, *368*(6486), 89–94. https://doi.org/10.1126/science.aaz9468

Dziedzic, N., Ho, A., Adabi, B., Foilb, A. R., & Romeo, R. D. (2014). Shifts in hormonal stress reactivity during adolescence are not associated with changes in glucocorticoid receptor levels in the brain and pituitary of male rats. *Developmental Neuroscience*, *36*(3–4), 261–268. https://doi.org/10.1159/000362873

Ellenbroek, B., & Youn, J. (2016). Rodent models in neuroscience research: Is it a rat race? *Disease Models & Mechanisms*, *9*(10), 1079–1087. https://doi.org/10.1242/dmm.026120

Essex, M. J., Boyce, W. T., Hertzman, C., Lam, L. L., Armstrong, J. M., Neumann, S. M. A., & Kobor, M. S. (2013). Epigenetic vestiges of early developmental adversity: Childhood stress exposure and DNA methylation in adolescence. *Child Development*, *84*(1), 58–75. https://doi.org/10.1111/j.1467-8624.2011.01641.x

Farrell, C., & O'Keane, V. (2016). Epigenetics and the glucocorticoid receptor: A review of the implications in depression. *Psychiatry Research*, *242*, 349–356. https://doi.org/10.1016/j.psychres.2016.06.022

Flanagan, J. M. (2015). Epigenome-wide association studies (EWAS): Past, present, and future. *Methods in Molecular Biology (Clifton, N.J.)*, *1238*, 51–63. https://doi.org/10.1007/978-1-4939-1804-1_3

Fraser, A., Macdonald-Wallis, C., Tilling, K., Boyd, A., Golding, J., Davey Smith, G., Henderson, J., Macleod, J., Molloy, L., Ness, A., Ring, S., Nelson, S. M., & Lawlor, D. A. (2013). Cohort Profile: The Avon Longitudinal Study of Parents and Children: ALSPAC mothers cohort. *International Journal of Epidemiology*, *42*(1), 97–110. https://doi.org/10.1093/ije/dys066

Freund, N., Thompson, B. S., Denormandie, J., Vaccarro, K., & Andersen, S. L. (2013). Windows of vulnerability: Maternal separation, age, and fluoxetine on adolescent depressive-like behavior in rats. *Neuroscience*, *249*, 88–97. https://doi.org/10.1016/j.neuroscience.2013.06.066

Frodl, T., & O'Keane, V. (2013). How does the brain deal with cumulative stress? A review with focus on developmental stress, HPA axis function and hippocampal structure in humans. *Neurobiology of Disease*, *52*, 24–37. https://doi.org/10.1016/j.nbd.2012.03.012

Galván, A. (2010). Adolescent Development of the Reward System. *Frontiers in Human Neuroscience*, *4*. https://doi.org/10.3389/neuro.09.006.2010

Galván, A. (2017). Adolescence, brain maturation and mental health. *Nature Neuroscience*, *20*(4), 503–504. https://doi.org/10.1038/nn.4530

Gee, D. G., Humphreys, K. L., Flannery, J., Goff, B., Telzer, E. H., Shapiro, M., Hare, T. A., Bookheimer, S. Y., & Tottenham, N. (2013). A Developmental Shift from Positive to Negative Connectivity in Human Amygdala–Prefrontal Circuitry. *Journal of Neuroscience*, *33*(10), 4584–4593. https://doi.org/10.1523/JNEUROSCI.3446-12.2013

Geronazzo-Alman, L., Eisenberg, R., Shen, S., Duarte, C. S., Musa, G. J., Wicks, J., Fan, B., Doan, T., Guffanti, G., Bresnahan, M., & Hoven, C. W. (2017). Cumulative Exposure to Work-related Traumatic Events and Current Post-traumatic Stress Disorder in New York City's First Responders. *Comprehensive Psychiatry*, *74*, 134–143. https://doi.org/10.1016/j.comppsych.2016.12.003

Giedd, J. N., Snell, J. W., Lange, N., Rajapakse, J. C., Casey, B. J., Kozuch, P. L., Vaituzis, A. C., Vauss, Y. C., Hamburger, S. D., Kaysen, D., & Rapoport, J. L. (1996). Quantitative magnetic resonance imaging of human brain development: Ages 4-18. *Cerebral Cortex (New York, N.Y.: 1991)*, *6*(4), 551–560. https://doi.org/10.1093/cercor/6.4.551

Gildawie, K. R., Honeycutt, J. A., & Brenhouse, H. C. (2020). Region-specific Effects of Maternal Separation on Perineuronal Net and Parvalbumin-expressing Interneuron Formation in Male and Female Rats. *Neuroscience*, *428*, 23–37. https://doi.org/10.1016/j.neuroscience.2019.12.010

Global Burden of Disease Study 2013 Collaborators. (2015). Global, regional, and national incidence, prevalence, and years lived with disability for 301 acute and chronic diseases and injuries in 188 countries, 1990-2013: A systematic analysis for the Global Burden of Disease Study 2013. *Lancet (London, England)*, *386*(9995), 743–800. https://doi.org/10.1016/S0140-6736(15)60692-4

Gogtay, N., Giedd, J. N., Lusk, L., Hayashi, K. M., Greenstein, D., Vaituzis, A. C., Nugent, T. F., Herman, D. H., Clasen, L. S., Toga, A. W., Rapoport, J. L., & Thompson, P. M. (2004). Dynamic mapping of human cortical development during childhood through early adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(21), 8174–8179. https://doi.org/10.1073/pnas.0402680101

Hannon, E., Lunnon, K., Schalkwyk, L., & Mill, J. (2015). Interindividual methylomic variation across blood, cortex, and cerebellum: Implications for epigenetic studies of neurological and neuropsychiatric phenotypes. *Epigenetics*, *10*(11), 1024–1032. https://doi.org/10.1080/15592294.2015.1100786

Hart, H., & Rubia, K. (2012). Neuroimaging of child abuse: A critical review. *Frontiers in Human Neuroscience*, *6*. https://doi.org/10.3389/fnhum.2012.00052

Heim, C., Newport, D. J., Mletzko, T., Miller, A. H., & Nemeroff, C. B. (2008). The link between childhood trauma and depression: Insights from HPA axis studies in humans. *Psychoneuroendocrinology*. https://doi.org/10.1016/j.psyneuen.2008.03.008

Hoehe, M. R., & Morris-Rosendahl, D. J. (2018). The role of genetics and genomics in clinical psychiatry. *Dialogues in Clinical Neuroscience*, *20*(3), 169–177.

Honeycutt, J. A., Demaestri, C., Peterzell, S., Silveri, M. M., Cai, X., Kulkarni, P., Cunningham, M. G., Ferris, C. F., & Brenhouse, H. C. (2020). Altered corticolimbic connectivity reveals sex-specific adolescent outcomes in a rat model of early life adversity. *ELife*, *9*. https://doi.org/10.7554/eLife.52651

Houston, S. M., Herting, M. M., & Sowell, E. R. (2014). The Neurobiology of Childhood Structural Brain Development: Conception Through Adulthood. *Current Topics in Behavioral Neurosciences*, *16*, 3–17. https://doi.org/10.1007/7854_2013_265

Jaddoe, V. W. V., Bakker, R., van Duijn, C. M., van der Heijden, A. J., Lindemans, J., Mackenbach, J. P., Moll, H. A., Steegers, E. A. P., Tiemeier, H., Uitterlinden, A. G., Verhulst, F. C., & Hofman, A. (2007). The Generation R Study Biobank: A resource for epidemiological studies in children and their parents. *European Journal of Epidemiology*, *22*(12), 917–923. https://doi.org/10.1007/s10654-007-9209-z

Jaenisch, R., & Bird, A. (2003). Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nature Genetics*, *33 Suppl*, 245–254. https://doi.org/10.1038/ng1089

Kapoor, A., Dunn, E., Kostaki, A., Andrews, M. H., & Matthews, S. G. (2006). Fetal programming of hypothalamo-pituitary-adrenal function: Prenatal stress and glucocorticoids. *The Journal of Physiology*, *572*(Pt 1), 31–44. https://doi.org/10.1113/jphysiol.2006.105254

Karsli-Ceppioglu, S. (2016). Epigenetic Mechanisms in Psychiatric Diseases and Epigenetic Therapy. *Drug Development Research*, *77*(7), 407–413. https://doi.org/10.1002/ddr.21340

Kember, R. L., Dempster, E. L., Lee, T. H. A., Schalkwyk, L. C., Mill, J., & Fernandes, C. (2012). Maternal separation is associated with strain-specific responses to stress and

epigenetic alterations to Nr3c1, Avp, and Nr4a1 in mouse. *Brain and Behavior*, *2*(4), 455–467. https://doi.org/10.1002/brb3.69

Kendler, K. S., Kuhn, J. W., & Prescott, C. A. (2004). Childhood sexual abuse, stressful life events and risk for major depression in women. *Psychological Medicine*, *null*(08), 1475–1482. https://doi.org/10.1017/S003329170400265X

Kessler, R. C., Avenevoli, S., & Ries Merikangas, K. (2001). Mood disorders in children and adolescents: An epidemiologic perspective. *Biological Psychiatry*, *49*(12), 1002–1014. https://doi.org/10.1016/S0006-3223(01)01129-5

Kessler, R. C., Berglund, P., Demler, O., Jin, R., Merikangas, K. R., & Walters, E. E. (2005). Lifetime prevalence and age-of-onset distributions of DSM-IV disorders in the National Comorbidity Survey Replication. *Archives of General Psychiatry*, *62*(6), 593–602. https://doi.org/10.1001/archpsyc.62.6.593

Klengel, T., Pape, J., Binder, E. B., & Mehta, D. (2014). The role of DNA methylation in stress-related psychiatric disorders. *Neuropharmacology*, *80*, 115–132. https://doi.org/10.1016/j.neuropharm.2014.01.013

Kolb, B., & Gibb, R. (2011). Brain Plasticity and Behaviour in the Developing Brain. *Journal of the Canadian Academy of Child and Adolescent Psychiatry*, *20*(4), 265–276.

Kooijman, M. N., Kruithof, C. J., van Duijn, C. M., Duijts, L., Franco, O. H., van IJzendoorn, M. H., de Jongste, J. C., Klaver, C. C. W., van der Lugt, A., Mackenbach, J. P., Moll, H. A., Peeters, R. P., Raat, H., Rings, E. H. H. M., Rivadeneira, F., van der Schroeff, M. P., Steegers, E. A. P., Tiemeier, H., Uitterlinden, A. G., … Jaddoe, V. W. V. (2016). The Generation R Study: Design and cohort update 2017. *European Journal of Epidemiology*, *31*(12), 1243–1264. https://doi.org/10.1007/s10654-016-0224-9

Koolschijn, P. C. M. P., & Crone, E. A. (2013). Sex differences and structural brain maturation from childhood to early adulthood. *Developmental Cognitive Neuroscience*, *5*, 106–118. https://doi.org/10.1016/j.dcn.2013.02.003

Koopman-Verhoeff, M. E., Mulder, R. H., Saletin, J. M., Reiss, I., Horst, G. T. J. van der, Felix, J. F., Carskadon, M. A., Tiemeier, H., & Cecil, C. A. M. (2020). Genome-wide DNA methylation patterns associated with sleep and mental health in children: A population-based study. *Journal of Child Psychology and Psychiatry*, *n/a*(n/a). https://doi.org/10.1111/jcpp.13252

Kular, L., & Kular, S. (2018). Epigenetics applied to psychiatry: Clinical opportunities and future challenges. *Psychiatry and Clinical Neurosciences*, *72*(4), 195–211. https://doi.org/10.1111/pcn.12634

Lagercrantz, H. (2016). Connecting the brain of the child from synapses to screen-based activity. *Acta Paediatrica (Oslo, Norway: 1992)*, *105*(4), 352–357. https://doi.org/10.1111/apa.13298

Lazarus, R. S. (1974). Psychological Stress and Coping in Adaptation and Illness. *The International Journal of Psychiatry in Medicine*, *5*(4), 321–333. https://doi.org/10.2190/T43T-84P3-QDUR-7RTP

Lehmann, J., & Feldon, J. (2000). Long-term biobehavioral effects of maternal separation in the rat: Consistent or confusing? *Reviews in the Neurosciences*, *11*(4), 383–408.

Lenroot, R. K., Gogtay, N., Greenstein, D. K., Wells, E. M., Wallace, G. L., Clasen, L. S., Blumenthal, J. D., Lerch, J., Zijdenbos, A. P., Evans, A. C., Thompson, P. M., & Giedd, J. N. (2007). Sexual Dimorphism of Brain Developmental Trajectories during Childhood and Adolescence. *NeuroImage*, *36*(4), 1065–1073. https://doi.org/10.1016/j.neuroimage.2007.03.053

Lenz, K. M., Nugent, B. M., Haliyur, R., & McCarthy, M. M. (2013). Microglia Are Essential to Masculinization of Brain and Behavior. *Journal of Neuroscience*, *33*(7), 2761–2772. https://doi.org/10.1523/JNEUROSCI.1268-12.2013

Leussis, M. P., Freund, N., Brenhouse, H. C., Thompson, B. S., & Andersen, S. L. (2012). Depressive-like behavior in adolescents after maternal separation: Sex differences, controllability, and GABA. *Developmental Neuroscience*, *34*(2–3), 210–217. https://doi.org/10.1159/000339162

Liu, C., Marioni, R. E., Hedman, Å. K., Pfeiffer, L., Tsai, P.-C., Reynolds, L. M., Just, A. C., Duan, Q., Boer, C. G., Tanaka, T., Elks, C. E., Aslibekyan, S., Brody, J. A., Kühnel, B., Herder, C., Almli, L. M., Zhi, D., Wang, Y., Huan, T., … Levy, D. (2018). A DNA methylation biomarker of alcohol consumption. *Molecular Psychiatry*, *23*(2), 422–433. https://doi.org/10.1038/mp.2016.192

Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P. M., & Meaney, M. J. (1997). Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. *Science*, *277*(5332), 1659–1662.

Lukkes, J. L., Mokin, M. V., Scholl, J. L., & Forster, G. L. (2009). Adult rats exposed to early-life social isolation exhibit increased anxiety and conditioned fear behavior, and altered hormonal stress responses. *Hormones and Behavior*, *55*(1), 248–256. https://doi.org/10.1016/j.yhbeh.2008.10.014

Lupien, S. J., McEwen, B. S., Gunnar, M. R., & Heim, C. (2009). Effects of stress throughout the lifespan on the brain, behaviour and cognition. *Nature Reviews. Neuroscience*, *10*(6), 434–445. https://doi.org/10.1038/nrn2639

Mahgoub, M., & Monteggia, L. M. (2013). Epigenetics and psychiatry. *Neurotherapeutics: The Journal of the American Society for Experimental NeuroTherapeutics*, *10*(4), 734–741. https://doi.org/10.1007/s13311-013-0213-6

Malykhin, N. V., Carter, R., Seres, P., & Coupland, N. J. (2010). Structural changes in the hippocampus in major depressive disorder: Contributions of disease and treatment. *Journal of Psychiatry & Neuroscience : JPN*, *35*(5), 337–343. https://doi.org/10.1503/jpn.100002

Maniam, J., & Morris, M. J. (2010). Long-term postpartum anxiety and depression-like behavior in mother rats subjected to maternal separation are ameliorated by palatable high fat diet. *Behavioural Brain Research*. https://doi.org/10.1016/j.bbr.2009.11.005

Maron, E., & Nutt, D. (2017). Biological markers of generalized anxiety disorder. *Dialogues in Clinical Neuroscience*, *19*(2), 147–158.

Martínez-Cerdeño, V., & Noctor, S. C. (2018). Neural Progenitor Cell Terminology. *Frontiers in Neuroanatomy*, *12*. https://doi.org/10.3389/fnana.2018.00104

Matosin, N., Cruceanu, C., & Binder, E. B. (2017). Preclinical and Clinical Evidence of DNA Methylation Changes in Response to Trauma and Chronic Stress. *Chronic Stress (Thousand Oaks, Calif.)*, *1*. https://doi.org/10.1177/2470547017710764

Mc Elroy, S., & Hevey, D. (2014). Relationship between adverse early experiences, stressors, psychosocial resources and wellbeing. *Child Abuse & Neglect*, *38*(1), 65–75. https://doi.org/10.1016/j.chiabu.2013.07.017

McCormick, C. M., & Mathews, I. Z. (2007). *HPA function in adolescence: Role of sex hormones in its regulation and the enduring consequences of exposure to stressors*. https://doi.org/10.1016/j.pbb.2006.07.012

McCoy, C. R., Rana, S., Stringfellow, S. A., Day, J. J., Wyss, J. M., Clinton, S. M., & Kerman, I. A. (2016). Neonatal maternal separation stress elicits lasting DNA methylation changes in the hippocampus of stress-reactive Wistar Kyoto rats. *The European Journal of Neuroscience*, *44*(10), 2829–2845. https://doi.org/10.1111/ejn.13404

Mendle, J., Leve, L. D., Ryzin, M. V., Natsuaki, M. N., & Ge, X. (2011). Associations Between Early Life Stress, Child Maltreatment, and Pubertal Development Among Girls in Foster Care. *Journal of Research on Adolescence*, *21*(4), 871–880. https://doi.org/10.1111/j.1532-7795.2011.00746.x

Meredith, R. M. (2015). Sensitive and critical periods during neurotypical and aberrant neurodevelopment: A framework for neurodevelopmental disorders. *Neuroscience and Biobehavioral Reviews*, *50*, 180–188. https://doi.org/10.1016/j.neubiorev.2014.12.001

Minshew, N. J., & Williams, D. L. (2007). The New Neurobiology of Autism: Cortex, Connectivity, and Neuronal Organization. *Archives of Neurology*, *64*(7), 945–950. https://doi.org/10.1001/archneur.64.7.945

Moffett, M. C., Harley, J., Francis, D., Sanghani, S. P., Davis, W. I., & Kuhar, M. J. (2006). Maternal Separation and Handling Affects Cocaine Self- Administration in Both the Treated Pups as Adults and the Dams. *Pharmacology*, *317*(3), 1210–1218. https://doi.org/10.1124/jpet.106.101139

Mundorf, A., & Freund, N. (2019). Early Life Stress and DNA Methylation. In S. Jurga & J. Barciszewski (Eds.), *The DNA, RNA, and Histone Methylomes* (pp. 215–227). Springer International Publishing. https://doi.org/10.1007/978-3-030-14792-1_9

Mundorf, A., Knorr, A., Mezö, C., Klein, C., Beyer, D. K., Fallgatter, A. J., Schwarz, M., & Freund, N. (2019). Lithium and glutamine synthetase: Protective effects following stress. *Psychiatry Research*, *281*, 112544. https://doi.org/10.1016/j.psychres.2019.112544

Mundorf, A., Rommel, S., Verheyen, M., Mergia, E., Peters, M., & Freund, N. (2020). Cigarette smoke exposure has region-specific effects on GDAP1 expression in mouse hippocampus. *Psychiatry Research*, 112979. https://doi.org/10.1016/j.psychres.2020.112979

Mundorf, A., Schmitz, J., Güntürkün, O., Freund, N., & Ocklenburg, S. (2018). Methylation of MORC1: A possible biomarker for depression? *Journal of Psychiatric Research*, *103*, 208–211. https://doi.org/10.1016/j.jpsychires.2018.05.026

Murgatroyd, C., Patchev, A. V., Wu, Y., Micale, V., Bockmühl, Y., Fischer, D., Holsboer, F., Wotjak, C. T., Almeida, O. F. X., & Spengler, D. (2009). Dynamic DNA methylation programs persistent adverse effects of early-life stress. *Nature Neuroscience*, *12*(12), 1559–1566. https://doi.org/10.1038/nn.2436

Nadarajah, B., Brunstrom, J. E., Grutzendler, J., Wong, R. O., & Pearlman, A. L. (2001). Two modes of radial migration in early development of the cerebral cortex. *Nature Neuroscience*, *4*(2), 143–150. https://doi.org/10.1038/83967

Nederhof, E., & Schmidt, M. V. (2012). Mismatch or cumulative stress: Toward an integrated hypothesis of programming effects. *Physiology & Behavior*, *106*(5), 691–700. https://doi.org/10.1016/j.physbeh.2011.12.008

Neufang, S., Specht, K., Hausmann, M., Güntürkün, O., Herpertz-Dahlmann, B., Fink, G. R., & Konrad, K. (2009). Sex differences and the impact of steroid hormones on the developing human brain. *Cerebral Cortex (New York, N.Y.: 1991)*, *19*(2), 464–473. https://doi.org/10.1093/cercor/bhn100

Nielsen, D. A., Utrankar, A., Reyes, J. A., Simons, D. D., & Kosten, T. R. (2012). Epigenetics of drug abuse: Predisposition or response. *Pharmacogenomics*, *13*(10), 1149–1160. https://doi.org/10.2217/pgs.12.94

Nieto, S. J., Patriquin, M. A., Nielsen, D. A., & Kosten, T. A. (2016). Don't worry; be informed about the epigenetics of anxiety. *Pharmacology, Biochemistry, and Behavior*, *146–147*, 60–72. https://doi.org/10.1016/j.pbb.2016.05.006

Oakes, P., Loukas, M., Oskouian, R. J., & Tubbs, R. S. (2017). The neuroanatomy of depression: A review. *Clinical Anatomy (New York, N.Y.)*, *30*(1), 44–49. https://doi.org/10.1002/ca.22781

Orso, R., Creutzberg, K. C., Wearick-Silva, L. E., Wendt Viola, T., Tractenberg, S. G., Benetti, F., & Grassi-Oliveira, R. (2019). How Early Life Stress Impact Maternal Care: A Systematic Review of Rodent Studies. *Frontiers in Behavioral Neuroscience*, *13*. https://doi.org/10.3389/fnbeh.2019.00197

Orso, R., Wearick-Silva, L. E., Creutzberg, K. C., Centeno-Silva, A., Glusman Roithmann, L., Pazzin, R., Tractenberg, S. G., Benetti, F., & Grassi-Oliveira, R. (2018). Maternal behavior of the mouse dam toward pups: Implications for maternal separation model of early life stress. *Stress*. https://doi.org/10.1080/10253890.2017.1389883

Ota, M., Noda, T., Sato, N., Hidese, S., Teraishi, T., Setoyama, S., Sone, D., Matsuda, H., & Kunugi, H. (2018). The use of diffusional kurtosis imaging and neurite orientation dispersion and density imaging of the brain in major depressive disorder. *Journal of Psychiatric Research*, *98*, 22–29. https://doi.org/10.1016/j.jpsychires.2017.12.011

Ovenden, E. S., McGregor, N. W., Emsley, R. A., & Warnich, L. (2018). DNA methylation and antipsychotic treatment mechanisms in schizophrenia: Progress and future directions.

Progress in Neuro-Psychopharmacology & Biological Psychiatry, *81*, 38–49. https://doi.org/10.1016/j.pnpbp.2017.10.004

Palazidou, E. (2012). The neurobiology of depression. *British Medical Bulletin*, *101*, 127–145. https://doi.org/10.1093/bmb/lds004

Palma-Gudiel, H., & Fañanás, L. (2017). An integrative review of methylation at the serotonin transporter gene and its dialogue with environmental risk factors, psychopathology and 5-HTTLPR. *Neuroscience and Biobehavioral Reviews*, *72*, 190–209. https://doi.org/10.1016/j.neubiorev.2016.11.011

Pariante, C. M., & Lightman, S. L. (2008). The HPA axis in major depression: Classical theories and new developments. *Trends in Neurosciences*, *31*(9), 464–468. https://doi.org/10.1016/j.tins.2008.06.006

Paternain, L., Martisova, E., Campión, J., Martínez, J. A., Ramírez, M. J., & Milagro, F. I. (2016). Methyl donor supplementation in rats reverses the deleterious effect of maternal separation on depression-like behaviour. *Behavioural Brain Research*, *299*, 51–58. https://doi.org/10.1016/j.bbr.2015.11.031

Paus, T. (2010). Growth of white matter in the adolescent brain: Myelin or axon? *Brain and Cognition*, *72*(1), 26–35. https://doi.org/10.1016/j.bandc.2009.06.002

Paus, T., Collins, D. L., Evans, A. C., Leonard, G., Pike, B., & Zijdenbos, A. (2001). Maturation of white matter in the human brain: A review of magnetic resonance studies. *Brain Research Bulletin*, *54*(3), 255–266. https://doi.org/10.1016/s0361-9230(00)00434-2

Peedicayil, J. (2012). Role of epigenetics in pharmacotherapy, psychotherapy and nutritional management of mental disorders. *Journal of Clinical Pharmacy and Therapeutics*, *37*(5), 499–501. https://doi.org/10.1111/j.1365-2710.2012.01346.x

Peña, C. J., Kronman, H. G., Walker, D. M., Cates, H. M., Bagot, R. C., Purushothaman, I., Issler, O., Loh, Y.-H. E., Leong, T., Kiraly, D. D., Goodman, E., Neve, R. L., Shen, L., & Nestler, E. J. (2017). Early life stress confers lifelong stress susceptibility in mice via ventral tegmental area OTX2. *Science (New York, N.Y.)*, *356*(6343), 1185–1188. https://doi.org/10.1126/science.aan4491

Peper, J. S., Brouwer, R. M., Schnack, H. G., van Baal, G. C., van Leeuwen, M., van den Berg, S. M., Delemarre-Van de Waal, H. A., Boomsma, D. I., Kahn, R. S., & Hulshoff Pol, H. E. (2009). Sex steroids and brain structure in pubertal boys and girls. *Psychoneuroendocrinology*, *34*(3), 332–342. https://doi.org/10.1016/j.psyneuen.2008.09.012

Philibert, R. A., Plume, J. M., Gibbons, F. X., Brody, G. H., & Beach, S. R. H. (2012). The impact of recent alcohol use on genome wide DNA methylation signatures. *Frontiers in Genetics*, *3*, 54. https://doi.org/10.3389/fgene.2012.00054

Poulton, R., Moffitt, T. E., & Silva, P. A. (2015). The Dunedin Multidisciplinary Health and Development Study: Overview of the first 40 years, with an eye to the future. *Social Psychiatry and Psychiatric Epidemiology*, *50*(5), 679–693. https://doi.org/10.1007/s00127-015-1048-8

Ptak, C., & Petronis, A. (2008). Epigenetics and complex disease: From etiology to new therapeutics. *Annual Review of Pharmacology and Toxicology, 48,* 257–276. https://doi.org/10.1146/annurev.pharmtox.48.113006.094731

Qiu, J. (2006). Epigenetics: Unfinished symphony. *Nature, 441*(7090), 143–145. https://doi.org/10.1038/441143a

Radtke, K. M., Schauer, M., Gunter, H. M., Ruf-Leuschner, M., Sill, J., Meyer, A., & Elbert, T. (2015). Epigenetic modifications of the glucocorticoid receptor gene are associated with the vulnerability to psychopathology in childhood maltreatment. *Translational Psychiatry, 5*, e571. https://doi.org/10.1038/tp.2015.63

Rakic, P. (1972). Mode of cell migration to the superficial layers of fetal monkey neocortex. *The Journal of Comparative Neurology, 145*(1), 61–83. https://doi.org/10.1002/cne.901450105

Razin, A., & Cedar, H. (1991). DNA methylation and gene expression. *Microbiological Reviews, 55*(3), 451–458.

Razin, A., & Riggs, A. D. (1980). DNA methylation and gene function. *Science (New York, N.Y.), 210*(4470), 604–610.

Raznahan, A., Shaw, P., Lalonde, F., Stockman, M., Wallace, G. L., Greenstein, D., Clasen, L., Gogtay, N., & Giedd, J. N. (2011). How Does Your Cortex Grow? *The Journal of Neuroscience, 31*(19), 7174–7177. https://doi.org/10.1523/JNEUROSCI.0054-11.2011

Reichelt, A. C., Hare, D. J., Bussey, T. J., & Saksida, L. M. (2019). Perineuronal Nets: Plasticity, Protection, and Therapeutic Potential. *Trends in Neurosciences, 42*(7), 458–470. https://doi.org/10.1016/j.tins.2019.04.003

Rijlaarsdam, J., Cecil, C. A. M., Walton, E., Mesirow, M. S. C., Relton, C. L., Gaunt, T. R., McArdle, W., & Barker, E. D. (2017). Prenatal unhealthy diet, insulin-like growth factor 2 gene (IGF2) methylation, and attention deficit hyperactivity disorder symptoms in youth with early-onset conduct problems. *Journal of Child Psychology and Psychiatry, and Allied Disciplines, 58*(1), 19–27. https://doi.org/10.1111/jcpp.12589

Roberts, S., Keers, R., Breen, G., Coleman, J. R. I., Jöhren, P., Kepa, A., Lester, K. J., Margraf, J., Scheider, S., Teismann, T., Wannemüller, A., Eley, T. C., & Wong, C. C. Y. (2019). DNA methylation of FKBP5 and response to exposure-based psychological therapy. *American Journal of Medical Genetics. Part B, Neuropsychiatric Genetics: The Official Publication of the International Society of Psychiatric Genetics, 180*(2), 150–158. https://doi.org/10.1002/ajmg.b.32650

Roberts, S., Keers, R., Lester, K. J., Coleman, J. R., Breen, G., Arendt, K., Blatter-Meunier, J., Cooper, P., Creswell, C., & Fjermestad, K. (2015). HPA axis related genes and response to psychological therapies: Genetics and epigenetics. *Depression and Anxiety, 32*(12), 861–870.

Roberts, S., Lester, K. J., Hudson, J. L., Rapee, R. M., Creswell, C., Cooper, P. J., Thirlwall, K. J., Coleman, J. R. I., Breen, G., Wong, C. C. Y., & Eley, T. C. (2014). Serotonin transporter [corrected] methylation and response to cognitive behaviour therapy in children

with anxiety disorders. *Translational Psychiatry*, *4*, e444. https://doi.org/10.1038/tp.2014.83

Rolls, A., Shechter, R., London, A., Ziv, Y., Ronen, A., Levy, R., & Schwartz, M. (2007). Toll-like receptors modulate adult hippocampal neurogenesis. *Nature Cell Biology*, *9*(9), 1081–1088. https://doi.org/10.1038/ncb1629

Roth, T. L., Lubin, F. D., Funk, A. J., & Sweatt, J. D. (2009). LASTING EPIGENETIC INFLUENCE OF EARLY-LIFE ADVERSITY ON THE BDNF GENE. *Biological Psychiatry*, *65*(9), 760–769. https://doi.org/10.1016/j.biopsych.2008.11.028

Sadava, D., Hillis, D., Heller, H. C., & Hacker, S. (2019). *Purves Biologie* (J. Markl, Ed.; 10th ed.). Springer Spektrum. https://doi.org/10.1007/978-3-662-58172-8

Santarelli, S., Lesuis, S. L., Wang, X.-D., Wagner, K. V., Hartmann, J., Labermaier, C., Scharf, S. H., Müller, M. B., Holsboer, F., & Schmidt, M. V. (2014). Evidence supporting the match/mismatch hypothesis of psychiatric disorders. *European Neuropsychopharmacology: The Journal of the European College of Neuropsychopharmacology*, *24*(6), 907–918. https://doi.org/10.1016/j.euroneuro.2014.02.002

Santos-Rebouças, C. B., & Pimentel, M. M. G. (2007). Implication of abnormal epigenetic patterns for human diseases. *European Journal of Human Genetics: EJHG*, *15*(1), 10–17. https://doi.org/10.1038/sj.ejhg.5201727

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine Reviews*, *21*(1), 55–89. https://doi.org/10.1210/edrv.21.1.0389

Schiller, C. E., Meltzer-Brody, S., & Rubinow, D. R. (2015). The role of reproductive hormones in postpartum depression. *CNS Spectrums*, *20*(01), 48–59. https://doi.org/10.1017/S1092852914000480

Schoedl, A. F., Costa, M. C. P., Mari, J. J., Mello, M. F., Tyrka, A. R., Carpenter, L. L., & Price, L. H. (2010). The Clinical Correlates of Reported Childhood Sexual Abuse: An Association Between Age at Trauma Onset and Severity of Depression and PTSD in Adults. *Journal of Child Sexual Abuse*, *19*(2), 156–170. https://doi.org/10.1080/10538711003615038

Sharma, S., Kelly, T. K., & Jones, P. A. (2010). Epigenetics in cancer. *Carcinogenesis*, *31*(1), 27–36. https://doi.org/10.1093/carcin/bgp220

Shaw, P., Kabani, N. J., Lerch, J. P., Eckstrand, K., Lenroot, R., Gogtay, N., Greenstein, D., Clasen, L., Evans, A., Rapoport, J. L., Giedd, J. N., & Wise, S. P. (2008). Neurodevelopmental Trajectories of the Human Cerebral Cortex. *The Journal of Neuroscience*, *28*(14), 3586–3594. https://doi.org/10.1523/JNEUROSCI.5309-07.2008

Shin, J., Ming, G.-L., & Song, H. (2014). DNA modifications in the mammalian brain. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *369*(1652). https://doi.org/10.1098/rstb.2013.0512

Shors, T. J., Anderson, L., Curlik, D. M., & Nokia, S. (2012). Use it or lose it: How neurogenesis keeps the brain fit for learning. *Behavioural Brain Research*, *227*(2), 450–458. https://doi.org/10.1016/j.bbr.2011.04.023

Sisk, C. L., & Foster, D. L. (2004). The neural basis of puberty and adolescence. *Nature Neuroscience*, *7*(10), 1040–1047. https://doi.org/10.1038/nn1326

Suliman, S., Mkabile, S. G., Fincham, D. S., Ahmed, R., Stein, D. J., & Seedat, S. (2009). Cumulative effect of multiple trauma on symptoms of posttraumatic stress disorder, anxiety, and depression in adolescents. *Comprehensive Psychiatry*, *50*(2), 121–127. https://doi.org/10.1016/j.comppsych.2008.06.006

Sullivan, P. F., de Geus, E. J. C., Willemsen, G., James, M. R., Smit, J. H., Zandbelt, T., Arolt, V., Baune, B. T., Blackwood, D., Cichon, S., Coventry, W. L., Domschke, K., Farmer, A., Fava, M., Gordon, S. D., He, Q., Heath, A. C., Heutink, P., Holsboer, F., … Penninx, B. W. J. H. (2009). Genome-wide association for major depressive disorder: A possible role for the presynaptic protein piccolo. *Molecular Psychiatry*, *14*(4), 359–375. https://doi.org/10.1038/mp.2008.125

Sung, Y. H., Shin, M. S., Cho, S., Baik, H. H., Jin, B. K., Chang, H. K., Lee, E. K., & Kim, C. J. (2010). Depression-like state in maternal rats induced by repeated separation of pups is accompanied by a decrease of cell proliferation and an increase of apoptosis in the hippocampus. *Neuroscience Letters*, *470*(1), 86–90. https://doi.org/10.1016/j.neulet.2009.12.063

Sveinbjornsdottir, S. (2016). The clinical symptoms of Parkinson's disease. *Journal of Neurochemistry*, *139 Suppl 1*, 318–324. https://doi.org/10.1111/jnc.13691

Taki, Y., Hashizume, H., Thyreau, B., Sassa, Y., Takeuchi, H., Wu, K., Kotozaki, Y., Nouchi, R., Asano, M., Asano, K., Fukuda, H., & Kawashima, R. (2013). Linear and curvilinear correlations of brain gray matter volume and density with age using voxel-based morphometry with the Akaike information criterion in 291 healthy children. *Human Brain Mapping*, *34*(8), 1857–1871. https://doi.org/10.1002/hbm.22033

Teicher, M. H., Samson, J. A., Anderson, C. M., & Ohashi, K. (2016). The effects of childhood maltreatment on brain structure, function and connectivity. *Nature Reviews Neuroscience*, *17*(10), 652–666. https://doi.org/10.1038/nrn.2016.111

Thion, M. S., Ginhoux, F., & Garel, S. (2018). Microglia and early brain development: An intimate journey. *Science*, *362*(6411), 185–189. https://doi.org/10.1126/science.aat0474

Thomas, M., Knoblich, N., Wallisch, A., Glowacz, K., Becker-Sadzio, J., Gundel, F., Brückmann, C., & Nieratschker, V. (2018). Increased BDNF methylation in saliva, but not blood, of patients with borderline personality disorder. *Clinical Epigenetics*, *10*(1), 109. https://doi.org/10.1186/s13148-018-0544-6

Tottenham, N., & Galván, A. (2016). Stress and the Adolescent Brain: Amygdala-Prefrontal Cortex Circuitry and Ventral Striatum as Developmental Targets. *Neuroscience and Biobehavioral Reviews*, *70*, 217–227. https://doi.org/10.1016/j.neubiorev.2016.07.030

Tractenberg, S. G., Levandowski, M. L., de Azeredo, L. A., Orso, R., Roithmann, L. G., Hoffmann, E. S., Brenhouse, H., & Grassi-Oliveira, R. (2016). An overview of maternal separation effects on behavioural outcomes in mice: Evidence from a four-stage methodological systematic review. *Neuroscience and Biobehavioral Reviews*, *68*, 489–503. https://doi.org/10.1016/j.neubiorev.2016.06.021

Tyborowska, A., Volman, I., Niermann, H. C. M., Pouwels, J. L., Smeekens, S., Cillessen, A. H. N., Toni, I., & Roelofs, K. (2018). Early-life and pubertal stress differentially modulate grey matter development in human adolescents. *Scientific Reports*, *8*(1), 9201. https://doi.org/10.1038/s41598-018-27439-5

Ueno, M., Fujita, Y., Tanaka, T., Nakamura, Y., Kikuta, J., Ishii, M., & Yamashita, T. (2013). Layer V cortical neurons require microglial support for survival during postnatal development. *Nature Neuroscience*, *16*(5), 543–551. https://doi.org/10.1038/nn.3358

Verney, C., Monier, A., Fallet-Bianco, C., & Gressens, P. (2010). Early microglial colonization of the human forebrain and possible involvement in periventricular white-matter injury of preterm infants. *Journal of Anatomy*, *217*(4), 436–448. https://doi.org/10.1111/j.1469-7580.2010.01245.x

Vinkers, C. H., Kalafateli, A. L., Rutten, B. P. F., Kas, M. J., Kaminsky, Z., Turner, J. D., & Boks, M. P. M. (2015). Traumatic stress and human DNA methylation: A critical review. *Epigenomics*, *7*(4), 593–608. https://doi.org/10.2217/epi.15.11

Walton, E., Pingault, J.-B., Cecil, C. a. M., Gaunt, T. R., Relton, C. L., Mill, J., & Barker, E. D. (2017). Epigenetic profiling of ADHD symptoms trajectories: A prospective, methylome-wide study. *Molecular Psychiatry*, *22*(2), 250–256. https://doi.org/10.1038/mp.2016.85

Weaver, I. C. G., Cervoni, N., Champagne, F. A., D'Alessio, A. C., Sharma, S., Seckl, J. R., Dymov, S., Szyf, M., & Meaney, M. J. (2004). Epigenetic programming by maternal behavior. *Nature Neuroscience*, *7*(8), 847–854. https://doi.org/10.1038/nn1276

Weaver, I. C. G., Meaney, M. J., & Szyf, M. (2006). Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(9), 3480–3485. https://doi.org/10.1073/pnas.0507526103

Weiss, I. C., Pryce, C. R., Jongen-Rêlo, A. L., Nanz-Bahr, N. I., & Feldon, J. (2004). Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. *Behavioural Brain Research*, *152*(2), 279–295. https://doi.org/10.1016/j.bbr.2003.10.015

World Health Organization. (2018). *WHO | Depression*. WHO. http://www.who.int/mediacentre/factsheets/fs369/en/

Worlein, J. M. (2014). Nonhuman Primate Models of Depression: Effects of Early Experience and Stress. *ILAR Journal*, *55*(2), 259–273. https://doi.org/10.1093/ilar/ilu030

Xie, R., Xie, H., Daniel, K., & He, G. (2018). Plasma concentrations of neurotransmitters and postpartum depression. *Journal of Central South University (Medical Sciences)*, *43*(3), 274–281. https://doi.org/10.11817/j.issn.1672-7347.2018.03.007

Xu, L., Sun, Y., Gao, L., Cai, Y.-Y., & Shi, S.-X. (2014). Prenatal restraint stress is associated with demethylation of corticotrophin releasing hormone (CRH) promoter and enhances CRH transcriptional responses to stress in adolescent rats. *Neurochemical Research*, *39*(7), 1193–1198. https://doi.org/10.1007/s11064-014-1296-0

Yakovlev, P., Lecours, A.-R., Minkowski, A., & _Davis, F. (1967). *Regional development of the brain in early life*.

Zimmerman, M., McDermut, W., & Mattia, J. I. (2000). Frequency of anxiety disorders in psychiatric outpatients with major depressive disorder. *The American Journal of Psychiatry*, *157*(8), 1337–1340. https://doi.org/10.1176/appi.ajp.157.8.1337

Appendix

Appendix A: Declaration (Erklärung)

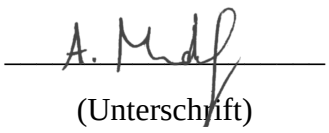
ERKLÄRUNG

Ich versichere an Eides statt, dass ich die eingereichte Dissertation selbstständig und ohne unzulässige fremde Hilfe verfasst, andere als die in ihr angegebene Literatur nicht benutzt und dass ich alle ganz oder annähernd übernommenen Textstellen sowie verwendete Grafi-ken und Tabellen kenntlich gemacht habe. Weiterhin erkläre ich, dass digitale Abbildungen nur die originalen Daten enthalten oder eine eindeutige Dokumentation von Art und Umfang der inhaltsverändernden Bildbearbeitung vorliegt. Außerdem versichere ich, dass es sich bei der von mir vorgelegten Dissertation (elektronische und gedruckte Version) um völlig übereinstimmende Exemplare handelt und die Dissertation in dieser oder ähnlicher Form noch nicht anderweitig als Promotionsleistung vorgelegt und bewertet wurde.

Es wurden keine anderen als die angegebenen Hilfsmittel verwendet.

Die Dissertation wurde gemäß der Promotionsordnung und der Betreuungsvereinbarung angefertigt.

Bochum, den 01.07.2020



(Unterschrift)

Appendix C: List of contribution to manuscripts

Declaration on the contribution to each manuscript

Chapter 2:

Abraham M, **Mundorf A**, Freund F (submitted). Unraveling the mystery of white matter alterations in depression: A comprehensive study of recent advances.
Review article
Own contribution: Planning: 60%, Writing of Manuscript: 30%

Chapter 3:

Mundorf A, Freund N (2019). Early life stress and DNA methylation. *In: The DNA, RNA, and Histone Methylomes., RNA Technologies. Springer International Publishing*.
Book chapter
Own contribution: Planning: 90%, Writing of Manuscript: 80%

Mundorf A, Bölükbas I, Freund N (submitted). Reduced ultrasonic vocalization in adolescent rats in a test for anxiety.
Research acticle
Own contribution: Planning: 80%, Experimental implementation: 90%, Writing of Manuscript: 90%, Figures: 100%

Mundorf A, Matsui H, Ocklenburg S and Freund F (Minor Revision/2020). Asymmetry of turning behavior in rats is modulated by early life stress. *Behav. Brain Res*.
Research article
Own contribution: Planning: 90%, Experimental implementation: 80%, Writing of Manuscript: 70%, Figures by SO

Mundorf A, Koch J, Kubitza N, Schmidt M, Gass P, Freund N (in preparation). Morc1 as a potential new target gene in mood regulation: When and where to find in the brain
Research article
Own contribution: Planning: 80%, Experimental implementation: 40%, Writing of Manuscript: 90%, Figures: 100%

Mundorf A, Freund N (in preparation). Morc1 RNA expression after early stress exposure during different developmental stages / sensitive windows
Research article
Own contribution: Planning: 80%, Experimental implementation: 100%, Writing of Manuscript: 90%, Figures: 100%

Chapter 4:

Bölükbas I*, **Mundorf A***, Freund N (submitted). Maternal separation effects on mothers – from rodents to insights in humans.
Review article *shared first
Own contribution: Planning: 50%, Writing of Manuscript: 40%, Tables: 50%

Bölükbas I, **Mundorf A**, Freund N (submitted). Maternal separation in rat dams – a neurobiological and behavioral approach to characterize the maternal side.
Research article
Own contribution: Planning: 70%, Experimental implementation: 30%, Writing of Manuscript: 40%, Figures: 30%

Ibrahim Bölükbas conducted the study as part of his medical doctoral thesis. I, therefore supervised him during experimental implementation and supported him with the analysis and writing of manuscript.

Chapter 5:
Mundorf A, Schmitz J, Güntürkün O, Freund N and Ocklenburg S (2018). Methylation of Morc1: A possible biomarker for depression?
Research article published (2018) J. Psychiatr. Res. 103, 208–211
Own contribution: Planning: 80%, Experimental implementation: 0%, Writing of Manuscript: 70%, Analysis of data by JS, Figures by JS

Mundorf A, Schmitz J, Hünten K, Fraenz C, Schlüter C, Genc E, Ocklenburg S and Freund N (in prepataion). MORC1 methylation and BDI are associated with microstructural features of the hippocampus and medial prefrontal cortex
Research article

Own contribution: Planning: 80%, Experimental implementation: 0%, Writing of Manuscript: 80%, Analysis of data by JS, CF, CS and GE. Figures by JS.

Mundorf A, Rommel S, Verheyen M, Mergia E, Peters M and Freund N (2020). Cigarette smoke exposure has region-specific effects on GDAP1 expression in mice hippocampus.
Research article published (2020) Psychiatry Res. July;289:112979
Own contribution: Planning: 50%, Experimental implementation: 30%, Writing of Manuscript: 80%, Figures: 100%
Stefanie Rommel conducted the study as part of her B.Sc. thesis and was supervised and supported during experimental implementation.

Mundorf A, Knorr A, Mezö C, Klein C, Beyer DK, Fallgatter AJ, Schwarz M, Freund N (2019). Lithium and glutamine synthetase: Protective effects following stress.
Research article published (2019) Psychiatry Res. 281, 112544.
Own contribution: Planning: 20%, Experimental implementation: 20%, Writing of Manuscript: 80%, Figures: 40%

Appendix D: Acknowledgments

Foremost, I would like to thank my first supervisor Jun.-Prof. Dr. Nadja Freund. She managed to supervise this thesis with an inspiring combination of support whenever I needed it and trust to enable me to develop into an independent researcher. Her unshakable enthusiasm for science and the great number of conversations we had about life, research, and scientific career management, has been inspiring to me throughout the last years. I am deeply thankful for her supervision, motivation, and support throughout all this time. Moreover, I am grateful to Prof. Dr. Stefan Herlitze for being my second supervisor. Further, I want to thank PD Dr. Sebastian Ocklenburg for the fruitful and exciting collaboration. I am grateful for all the fascinating projects and the friendship that emerged from it.

Furthermore, I had the pleasure to work in the best lab I could have wished for. Thus, I am grateful to the whole experimental and molecular psychiatry lab for providing a motivational, familiar, and positive atmosphere during and after work. Special thanks to Jenny, Dominik, and Marie-Pierre who always had a cup of coffee and an open ear whenever I needed it. Also, I want to thank all the students I have supervised during this time and who have provided helpful hands during animal experiments and have renewed my motivation and inspiration with all the little chats about science we had. I want to give great thanks to Stephi, Erica, Judith, and Lukas for their friendship, inspiring conversations, and their constant mental support when research wasn’t working as expected.

Lastly, I want to thank my family and friends for always supporting my decision for a scientific career, for understanding my lack of time in stressful periods, and for keeping on pushing me to “finally finish” my PhD.

Appendix E: Supplementary material

Chapter 4: Maternal separation effects on mothers – from rodents to insights in humans

Supplement Tables

TABLE 1.1. Study Characteristics and outcomes on maternal behavior. Increased, decreased and unaltered maternal care were obtained from data in rodents (mice and rats) after brief and long maternal separation (BMS and LMS) from pups. Comparisons with human findings of women suffering from postpartum depression demonstrate the similarities that occur in dams after BMS and LMS with affected women.

Apendix

Maternal separation outcomes		BMS References		LMS References		References (including reviews) with PPD confirmations in humans
		Rats	Mice	Rats	Mice	
Increased maternal behavior↑	More licking and grooming	(Boccia & Pedersen, 2001) (Liu et al., 1997) (Macrì, Chiarotti, & Würbel, 2008) (Macrì, Mason, & Würbel, 2004)	(Own & Patel, 2013) (Wei et al., 2010)	(Zimmerberg & Sageser, 2011) (Zimmerberg, Rosenthal, & Stark, 2003) (Macrì et al., 2008) (Macrì et al., 2004) (Neumann, Krömer, & Bosch, 2005) (Boccia et al., 2007)	(Own & Patel, 2013)	(Lovejoy & Graczyk, 2000) (Field, 2010) (Kim et al., 2014) (Weinberg et al., 2008) (M. O'Hara & McCabe, 2013) (Atkinson et al., 2000)
	More nest rebuilding/ quality and reorganizing behaviors	(Boccia & Pedersen, 2001)	(Own & Patel, 2013)	(Zimmerberg & Sageser, 2011) (Boccia & Pedersen, 2001)	(Own & Patel, 2013) (Millstein & Holmes, 2007) (strain differences enlisted)	
	More relocation of pups			(Zimmerberg & Sageser, 2011)		
	More pup retrieval			(Zimmerberg et al., 2003)		
	More active nursing (compared to passive supine)	(Pryce et al., 2001) (Macrì et al., 2004) (Liu et al., 1997)	(D'Amato & Cabib, 1987; D'Amato, Cabib, Ventura, & Orsini, 1998) (Own & Patel, 2013)	(Zimmerberg & Sageser, 2011) (Macrì et al., 2008) (Macrì et al., 2004)	(Own & Patel, 2013)	
	More time on nest/ time spent with pup		(D'Amato & Cabib, 1987; (Own & Patel, 2013)	(Zimmerberg & Sageser, 2011) (Macrì et al., 2004) (Zimmerberg, Rosenthal, & Stark, 2003)	(Own & Patel, 2013)	
No changes in maternal care↔	No differences on licking and grooming	(Pryce et al., 2001) (Boccia & Pedersen, 2001)	(Anisman et al., 1998) (D'Amato et al., 1998)	(Pryce et al., 2001)	(Romeo et al., 2003)	
	No changes in pup retrieval (time)			(Boccia & Pedersen, 2001)	Romeo et al., 2003)	
	No changes in self-grooming	(Macrì, Mason, & Würbel, 2004)	(D'Amato et al., 1998)	(Zimmerberg et al., 2003)		
	No changes in rearing			(Boccia & Pedersen, 2001)		
	No changes in crouching, carrying, regrouping	(Pryce et al., 2001)		(Boccia & Pedersen, 2001) (Pryce et al., 2001)		

	No difference on active nursing	(Macrì et al., 2008)	(Anisman et al., 1998) (Wei et al., 2010)	(Macrì et al., 2004)	(Romeo et al., 2003)	
	No differences time off the nest		(Anisman et al., 1998)	(Zimmerberg et al., 2003) (Neumann, Wigger, et al., 2005)	Romeo et al., 2003)	
	No change in nest-building	(Boccia & Pedersen, 2001)		(Boccia & Pedersen, 2001)	(Romeo et al., 2003)	
Less maternal care↓	Decreased licking and or grooming			(Boccia et al., 2007) (Boccia & Pedersen, 2001)		
	Less self-grooming		(D'Amato & Cabib, 1987)			
	Nest-building was impaired		(D'Amato et al., 1998)	(Aguggia, Suárez, & Rivarola, 2013)		
	Less offspring-directed behavior			(Aguggia et al., 2013) (Boccia & Pedersen, 2001)		
	Slower / less pup retrieval			(Aguggia et al., 2013) (Maniam & Morris, 2010)		
	More exploratory activity towards child (Increased number of rearings)	(Boccia & Pedersen, 2001)		(Aguggia et al., 2013)		

TABLE 1.2. Study Characteristics and outcomes on anxiety-like, depression-like behavior, aggression, changes in reward circuitry and memory. Increased, decreased and unaltered behavioral outcomes were obtained from data in rodents (mice and rats) after brief and long maternal separation (BMS and LMS) from pups. Comparisons with human findings of women suffering from postpartum depression demonstrate the similarities that occur in dams after BMS and LMS with affected women.

Maternal separation outcomes		**BMS References**		**LMS References**		**References (including reviews) with PPD confirmations in humans**
		Rats	**Mice**	**Rats**	**Mice**	
Anxiety↑	Elevated plus maze: Less time in open arm			(Bousalham, Benazzouz, Hessni, Ouichou, & Mesfioui, 2013) (Maniam & Morris, 2010)		
	Elevated plus maze: Less emtries open-arm			(Aguggia et al., 2013)		
	Open field test: Less time spent in central area			(Bousalham et al., 2013)		
	Multivariate concentric square field: Lower risk assessment behavior	(Daoura, 2010)				
	Less exploratory activity of environment	(Daoura, 2010)	(D'Amato et al., 1998)			
Anxiety↔	Elevated plus maze: no differences on time spent on open arms	(Stevenson et al., 2009) (Kalinichev, Easterling, & Holtzman, 2000)		(Stevenson et al., 2009) (Boccia & Pedersen, 2001) (Aguggia, Suárez, & Rivarola, 2013) (Aguggia et al., 2013)		
	Arm entries: no differences on open and closed arms entries			(Bousalham, Benazzouz, Hessni, Ouichou, & Mesfioui, 2013)		
	T-Maze: no difference on first choice			(Zimmerberg et al., 2003)		

	Defensive withdrawal test (latency time): no differences	(Eklund et al., 2009)		(Eklund et al., 2009)		
Anxiety ↓	Elevated plus maze: more time on open-arms and open-arm entries	(Kalinichev et al., 2000) (Boccia & Pedersen, 2001)		(Kalinichev et al., 2000) (Boccia & Pedersen, 2001)		
Depression-like behavior↑	Forced swim test: more time of immobility			(Boccia et al., 2007) (Maniam & Morris, 2010) (Bousalham et al., 2013) (Sung et al., 2010)		
	Sucrose test: less sucrose intake			(Maniam & Morris, 2010)		
Depression-like behavior↔	Forced swim test: no difference on time of immobility	(Boccia et al., 2007)		(Aguggia et al., 2013)		
	Sucrose test: no changes in sucrose intake	(Maniam & Morris, 2010)				
Aggression ↔	No alterations of aggression	(Boccia & Pedersen, 2001)				(Ou & Hall, 2018) (Walker, Davis, Al-Sahab, & Tamim, 2013)
Aggression↓	Less aggression	(Boccia & Pedersen, 2001)		(Boccia & Pedersen, 2001)		
Changes in reward circuitry system	Drug-abuse such as cocaine self-administration altered	(Moffett et al., 2006)		(Moffett et al., 2006)		(Field, 2010) (Josefsson, Larsson, Sydsjö, & Nylander, 2007) (Nielsen, Videbech, Hedegaard, Dalby, & Secher, 2000) (Wieck et al., 1991) (Moses-Kolko, 2010; Moses-Kolko et al., 2011, 2012)

	Reward-related USVs: more 50-kHz calls	(Stevenson et al., 2009) PND13		(Stevenson et al., 2009) PND2		
	Sucrose test: more sucrose intake	(Michaels & Holtzman, 2006) (Michaels & Holtzmann, 2007)		(Michaels & Holtzman, 2006) (Michaels & Holtzmann, 2007)		
Memory and maternal memory	Lower latency in step-down inhibitory avoidance			(Aguggia et al., 2013) (Sung et al., 2010)		(Almeida & Sweeney, 2012) (Williams et al., 2015)

TABLE 2. Study Characteristics and outcomes on hormonal changes and neurochemical alterations including neurotransmitter. Several systems crucial in sexual hormonal and stress regulations of postpartum rodent dams (mice and rats) after brief and long maternal separation (BMS and LMS) from pups. Comparisons with human findings of women suffering from postpartum depression demonstrate the similarities that occur in dams after BMS and LMS with affected women.

Maternal separation outcomes		**BMS References**		**LMS References**		**References (including reviews) with PPD confirmations in humans**
		Rats	**Mice**	**Rats**	**Mice**	
Cortisol	Increased Corticosterone in plasma	(Eklund et al., 2009)	(F. R. D'Amato et al., 1992)	(Maniam & Morris, 2010) (Bánky, Nagy, & Halász, 1994) (Zarrow, Schlein, Denenberg, & Cohen, 1972)		(Schiller, Meltzer-Brody, & Rubinow, 2015) (Seth, Lewis, & Galbally, 2016) (Hendrick, Altshuler, & Suri, 1998) (Serati, Redaelli, Buoli, & Altamura, 2016) (Janssen et al., 2016) (Yim, Ilona S. & Glynn, 2009)
	Decreased adrenal gland weight	(Eklund et al., 2009)				
	No changes in corticosterone in plasma		(Wei et al., 2010)			
	Decreased basal corticosterone levels in plasma	(Leuner, Mirescu, Noiman, & Elizabeth Gould, 2007)				
Prolactin	Decreased prolactin in plasma			(Bánky, Nagy, & Halász, 1994) (Zarrow et al., 1972)		(Cabrera-Reyes, Limón-Morales, Rivero-Segura, Camacho-Arroyo, & Cerbón, 2017)

						(Fitzgerald & Dinan, 2008) (Groer & Morgan, 2007a, 2007b) (Schiller et al., 2015) (T. Abou-Saleh, Ghubash, Karim, Krymski, & Bhai, 1998) (Larsen & Grattan, 2012)
Estrogen	Decreased reproductive function and fertility			(Bousalham et al., 2013)		(Schiller et al., 2015) (Hendrick et al., 1998)
Oxytocin	No differences in Oxytocin levels in plasma	(Eklund et al., 2009)				(Moura, Canavarro, & Figueiredo-Braga, 2016) (Zonana & Gorman, 2005)
Serotonin-System	Anxiolytic-like effect of 8–OH–DPAT (serotonin-agonist)			(Picazo, Rosenblatt, & Fernández-Guasti, 2000)		(Misri, Reebye, Corral, & Milis, 2004) (Aishwarya et al., 2013) (Maurer-Spurej et al., 2007)
	Decrease Serotonin (5-Hydroxytrymptamine) and tryptophan hydroxylase in dorsal raphe			(Sung et al., 2010)		(Frokjaer et al., 2015) (Anderson & Maes, 2013)
Endocannabinoid (ECS)- and Opioid system	Decreased sensitivity to antinociceptive qualities of morphine and hyperalgesia			(Kalinichev et al., 2000)		(Eisenach, 2008) (Yim et al., 2010) (Nelson, 1998) (Watkins, Meltzer-Brody, Zolnoun, & Stuebe, 2011)

TABLE 3. Study Characteristics and outcomes neuroanatomical alterations including gene expressions of postpartum rodent dams (mice and rats) after brief and long maternal separation (BMS and LMS) from pups. Comparisons with human findings of women suffering from postpartum depression demonstrate the similarities that occur in dams after BMS and LMS with affected women.

Maternal separation outcomes		**BMS References**		**LMS References**		**References (including reviews) with PPD confirmations in humans**
		Rats	**Mice**	**Rats**	**Mice**	
Hypothalamus	Increased Corticotropin-releasing Hormone mRNA			(Maniam & Morris, 2010)		(Yim, Ilona S. & Glynn, 2009) (placental CRH) (Magiakou et al., 1996) (placental CRH) (Kalantaridou et al., 2004) (placental CRH) (Bloch, 2003)
	Decreased Neuropeptide Y mRNA			(Maniam & Morris, 2010)		(Xie et al., 2018)
Pituitary gland	Adrenocorticotropic hormone in plasma: No differences detected	(Eklund et al., 2009)				(Yim, Ilona S. & Glynn, 2009)
Hippocampus	Prevented decrease in cell proliferation in hippocampus	(Leuner et al., 2007)				(Deligiannidis et al., 2013) (Brummelte & Galea, 2016) (Katz et al., 2012)
	Decrease of cell proliferation and increase of apoptosis			(Sung et al., 2010)		
	Glucocorticoid receptor mRNA: decreased			(Maniam & Morris, 2010)		
	Decreased Na, K-ATPase activity,NO-activity	(Von Poser Toigo et al., 2012)		(Von Poser Toigo et al., 2012)		
Amygdala	C-Fos: increased expression			(Aguggia et al., 2013)		(Deligiannidis et al., 2013) (Moses-Kolko et al., 2008)

Chapter 5: *MORC1* methylation and BDI are associated with microstructural features of the hippocampus and medial prefrontal cortex

Table S1: correlation coefficients

	FICVF hippocampus LH	FICVF hippocampus RH	ODI hippocampus LH	ODI hippocampus RH	ISO hippocampus LH	ISO hippocampus RH	Volume hippocampus LH	Volume hippocampus RH	FICVF mPFC	ODI mPFC	ISO mPFC	Volume mPFC	Surface mPFC	Thickness mPFC
BDI	0.114	0.121	0.353	0.288	0.097	0.353	-0.058	-0.220	0.059	-0.005	-0.078	-0.099	-0.052	-0.019
promoter	0.001	-0.048	0.041	0.036	0.184	0.013	-0.158	-0.072	-0.213	-0.280	-0.316	0.011	-0.035	0.109
cg16259931	0.053	0.062	-0.151	-0.065	0.222	0.105	0.050	0.307	0.119	-0.134	0.331	0.303	0.291	-0.050
cg19857541	0.099	0.187	0.012	-0.032	0.094	0.069	-0.159	-0.199	0.226	-0.013	0.165	0.049	0.084	-0.075
cg05148217	0.043	0.020	0.000	-0.003	0.063	-0.051	-0.163	-0.195	-0.031	-0.310	-0.054	0.177	0.039	0.298
cg23550947	0.020	0.096	0.115	-0.008	0.030	-0.157	0.020	-0.033	-0.110	-0.140	-0.004	-0.059	-0.141	0.166
cg26540808	0.115	0.134	0.123	-0.060	0.013	0.000	0.140	0.033	-0.090	-0.197	-0.233	0.209	0.171	0.108
cg25456186	-0.178	-0.152	-0.099	-0.185	-0.234	-0.143	-0.128	-0.147	-0.140	0.031	-0.156	-0.052	-0.160	0.286
cg18733433	0.101	0.041	0.046	-0.059	0.092	0.025	-0.032	-0.027	-0.072	-0.027	-0.385	0.082	0.064	0.055
cg04167867	-0.106	-0.151	-0.050	-0.177	-0.016	-0.160	-0.071	0.028	-0.260	0.041	-0.113	0.038	0.113	-0.069
cg07090057	-0.361	-0.472	-0.229	-0.171	-0.084	-0.107	-0.299	-0.206	-0.285	-0.282	-0.044	-0.342	-0.320	0.118
cg19748686	-0.057	-0.098	0.234	0.165	0.315	0.190	-0.287	-0.237	-0.084	-0.058	0.136	-0.142	-0.116	0.089
cg25092670	-0.078	-0.159	-0.068	-0.016	0.170	0.041	-0.260	-0.189	-0.216	-0.184	-0.157	-0.142	-0.158	0.031
cg02040012	0.062	0.104	0.120	0.223	0.024	-0.037	0.089	0.106	-0.102	-0.175	-0.255	-0.054	-0.084	-0.020
cg27175191	-0.120	-0.244	0.084	0.063	0.078	0.136	-0.160	-0.300	-0.142	0.073	-0.103	-0.135	-0.118	0.058

Table S2: *p* values

	FICVF hippocampus LH	FICVF hippocampus RH	ODI hippocampus LH	ODI hippocampus RH	ISO hippocampus LH	ISO hippocampus RH	Volume hippocampus LH	Volume hippocampus RH	FICVF mPFC	ODI mPFC	ISO mPFC	Volume mPFC	Surface mPFC	Thickness mPFC
BDI	0.422	0.394	0.010	0.039	0.494	0.010	0.682	0.117	0.677	0.971	0.585	0.486	0.713	0.896
promoter	0.993	0.738	0.773	0.798	0.193	0.926	0.262	0.611	0.130	0.044	0.023	0.940	0.805	0.443
cg16259931	0.707	0.663	0.284	0.647	0.113	0.458	0.726	0.027	0.401	0.345	0.016	0.029	0.036	0.724

cg19857541	0.486	0.184	0.933	0.820	0.509	0.628	0.260	0.156	0.108	0.929	0.242	0.728	0.552	0.598
cg05148217	0.764	0.886	0.998	0.982	0.659	0.717	0.248	0.165	0.825	0.026	0.703	0.211	0.784	0.032
cg23550947	0.886	0.498	0.418	0.958	0.832	0.265	0.886	0.814	0.438	0.321	0.975	0.680	0.318	0.240
cg26540808	0.416	0.343	0.386	0.671	0.925	0.999	0.321	0.814	0.527	0.161	0.097	0.137	0.226	0.445
cg25456186	0.208	0.283	0.485	0.190	0.095	0.313	0.366	0.298	0.321	0.829	0.269	0.713	0.257	0.040
cg18733433	0.476	0.774	0.748	0.677	0.518	0.861	0.823	0.850	0.611	0.847	0.005	0.561	0.653	0.697
cg04167867	0.453	0.285	0.724	0.209	0.908	0.257	0.619	0.842	0.063	0.771	0.425	0.791	0.427	0.627
cg07090057	0.009	0.000	0.102	0.226	0.555	0.451	0.031	0.143	0.041	0.043	0.755	0.013	0.021	0.405
cg19748686	0.688	0.488	0.095	0.242	0.023	0.177	0.039	0.091	0.552	0.685	0.337	0.315	0.414	0.532
cg25092670	0.583	0.261	0.634	0.912	0.228	0.776	0.063	0.179	0.124	0.192	0.267	0.315	0.263	0.826
cg02040012	0.661	0.464	0.398	0.112	0.868	0.795	0.528	0.454	0.470	0.214	0.068	0.702	0.553	0.890
cg27175191	0.399	0.081	0.552	0.657	0.582	0.337	0.256	0.031	0.316	0.609	0.469	0.339	0.406	0.682

Table S3: correlation coefficients for partial correlations

	FICVF hippocampus LH	FICVF hippocampus RH	ODI hippocampus LH	ODI hippocampus RH	ISO hippocampus LH	ISO hippocampus RH	Volume hippocampus LH	Volume hippocampus RH	FICVF mPFC	ODI mPFC	ISO mPFC	Volume mPFC	Surface mPFC	Thickness mPFC
BDI	0.132	0.149	0.358	0.329	0.096	0.369	-0.070	-0.261	0.060	-0.007	-0.076	-0.117	-0.059	-0.015
promoter	-0.037	-0.112	0.035	0.020	0.185	-0.014	-0.255	-0.155	-0.245	-0.275	-0.329	-0.059	-0.098	0.104
cg16259931	0.019	0.002	-0.163	-0.083	0.242	0.058	-0.053	0.237	0.156	-0.115	0.319	0.226	0.235	-0.095
cg19857541	0.110	0.237	0.004	-0.063	0.088	0.085	-0.174	-0.202	0.223	-0.025	0.187	0.105	0.127	-0.053
cg05148217	-0.064	-0.117	-0.030	-0.094	0.055	-0.117	-0.344	-0.379	-0.095	-0.310	-0.054	0.074	-0.076	0.319
cg23550947	0.069	0.154	0.148	0.068	0.049	-0.190	0.004	-0.110	-0.048	-0.121	-0.049	-0.152	-0.216	0.118
cg26540808	0.028	0.029	0.102	-0.153	0.008	-0.068	0.025	-0.115	-0.150	-0.191	-0.247	0.097	0.069	0.112
cg25456186	-0.147	-0.129	-0.075	-0.132	-0.225	-0.147	-0.133	-0.190	-0.079	0.050	-0.196	-0.079	-0.186	0.252
cg18733433	0.053	-0.024	0.025	-0.136	0.083	0.003	-0.098	-0.076	-0.133	-0.033	-0.377	0.058	0.032	0.080
cg04167867	-0.059	-0.137	-0.019	-0.111	0.004	-0.181	-0.082	-0.004	-0.202	0.071	-0.168	-0.001	0.118	-0.141
cg07090057	-0.171	-0.296	-0.171	0.046	-0.061	0.013	-0.081	0.013	-0.153	-0.313	-0.084	-0.165	-0.149	0.076
cg19748686	0.007	-0.032	0.261	0.255	0.325	0.238	-0.265	-0.203	-0.051	-0.061	0.134	-0.088	-0.061	0.083
cg25092670	-0.037	-0.138	-0.051	0.037	0.179	0.062	-0.269	-0.192	-0.195	-0.181	-0.171	-0.138	-0.149	0.013
cg02040012	-0.027	0.018	0.091	0.163	0.010	-0.075	0.021	0.062	-0.193	-0.186	-0.239	-0.135	-0.165	0.013
cg27175191	-0.069	-0.230	0.113	0.154	0.092	0.164	-0.143	-0.333	-0.091	0.084	-0.128	-0.137	-0.103	0.027

Table S4: *p* values for partial correlations

	FICVF hippocampus LH	FICVF hippocampus RH	ODI hippocampus LH	ODI hippocampus RH	ISO hippocampus LH	ISO hippocampus RH	Volume hippocampus LH	Volume hippocampus RH	FICVF mPFC	ODI mPFC	ISO mPFC	Volume mPFC	Surface mPFC	Thickness mPFC
BDI	0.361	0.303	0.011	0.020	0.506	0.008	0.627	0.067	0.680	0.962	0.601	0.420	0.686	0.916
promoter	0.798	0.437	0.810	0.889	0.198	0.922	0.074	0.281	0.086	0.053	0.020	0.682	0.499	0.472
cg16259931	0.896	0.991	0.258	0.567	0.090	0.688	0.712	0.098	0.278	0.427	0.024	0.115	0.101	0.510
cg19857541	0.447	0.097	0.977	0.665	0.542	0.557	0.226	0.159	0.120	0.865	0.194	0.467	0.378	0.717

cg05148217	0.660	0.419	0.837	0.517	0.702	0.419	0.014	0.007	0.513	0.028	0.710	0.609	0.601	0.024
cg23550947	0.634	0.287	0.305	0.637	0.734	0.187	0.981	0.449	0.740	0.404	0.737	0.291	0.132	0.416
cg26540808	0.847	0.844	0.480	0.290	0.957	0.640	0.865	0.427	0.299	0.183	0.084	0.501	0.633	0.440
cg25456186	0.307	0.370	0.606	0.360	0.117	0.308	0.358	0.187	0.584	0.730	0.172	0.585	0.195	0.078
cg18733433	0.716	0.867	0.865	0.345	0.567	0.985	0.498	0.600	0.356	0.819	0.007	0.689	0.827	0.580
cg04167867	0.683	0.344	0.894	0.441	0.980	0.210	0.572	0.975	0.161	0.622	0.242	0.993	0.414	0.327
cg07090057	0.235	0.037	0.234	0.750	0.675	0.928	0.577	0.926	0.289	0.027	0.564	0.254	0.301	0.601
cg19748686	0.960	0.824	0.068	0.074	0.021	0.096	0.063	0.158	0.725	0.673	0.352	0.546	0.675	0.567
cg25092670	0.798	0.341	0.725	0.800	0.213	0.669	0.059	0.181	0.174	0.208	0.234	0.340	0.303	0.926
cg02040012	0.852	0.901	0.530	0.258	0.946	0.605	0.886	0.667	0.180	0.196	0.094	0.352	0.253	0.928
cg27175191	0.635	0.109	0.434	0.286	0.525	0.256	0.322	0.018	0.529	0.563	0.377	0.342	0.477	0.853