MASTERARBEIT

Titel der Masterarbeit

„Maternal immune activation epigenetically regulates expression of the serotonin transporter in the adult offspring brain"

verfasst von

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ABSTRACT

Major depressive disorder (MDD) is one of the most debilitating diseases worldwide, yet the pathological mechanisms underlying this common mental illness are poorly understood and a large proportion of patients are not adequately treated. In recent years, involvement of the immune system has been proposed - both in acute depressive episodes and in terms of a potent environmental risk factor during neural development in form of maternal immune activation (MIA). Indeed, in a rodent model mimicking gestational infection by MIA through the administration of Poly(I:C) to the pregnant dam, depressive-like behaviour of adult offspring has been reported. However, the underlying molecular mechanisms are only starting to be elucidated. Increasing evidence points towards epigenetic mechanisms as central mediators of the impact of environmental influences on gene expression and consecutively brain structure and function. Here, we investigated the effect of MIA on molecular participants of epigenetic regulation with a special focus on the serotonin transporter (SERT), critically involved in the aetiology of MDD and pharmacological antidepressant treatment, as well as selected epigenetic markers in the hippocampus of MIA offspring. We found a reduction of histone acetylation specifically for H4 in hippocampal tissue of MIA offspring and a selective decrease in levels of histone deacetylases (HDACs) 2 and 9. Both SERT mRNA and protein expression and were significantly reduced in MIA offspring and a significant decrease in H3 acetylation as well as an

increase in H4 acetylation at the SERT promoter was observed by chromatin immunoprecipitation (ChIP). These findings support the notion that epigenetic mechanisms contribute to the environmental programming of brain development and behaviour by embedding the impact of the early life experiences on gene expression. Thus the data suggests that distinct hippocampal global and gene-specific histone acetylation patterns may ingrain the effects of MIA on SERT expression and depression-like behaviour later in life.

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Depression ist eine verbreitete psychiatrische Erkrankung, die für die betroffenen Personen schwer behindernd sein kann. Trotzdem sind die pathologischen Vorgänge, die dieser Störung unterliegen, noch weitgehend unbekannt und Patienten sprechen auf die Behandlung durch gängige Antidepressiva nicht immer an. Eine Theorie, die die Beteiligung des Immunsystems vorsieht, sowohl in der akuten Phase der Erkrankung als auch während der empfindlichen Entwicklungsphase des Gehirns, z.B. durch eine virale Infektion während der Schwangerschaft, hat in den letzten Jahren an Bedeutung gewonnen. Ein Mausmodell des letzteren Vorgangs, in dem eine mütterliche Immun-Aktivierung (MIA) durch Verabreichung von Poly(I:C) an die trächtige Maus bewirkt wird, hat kürzlich gezeigt, dass der erwachsene Nachwuchs einer so behandelten Maus depressionsähnliche Verhaltensmuster aufweist, wobei die verantwortlichen molekularen Mechanismen ungeklärt sind. Epigenetische Vorgänge und Faktoren stellen ideale Kandidaten dar, um diese Interaktion zwischen Umwelteinflüssen und Genexpression, und folglich auch deren Einfluss auf Gehirnstruktur und -funktion, zu vermitteln. In dieser Studie wurde der Einfluss von MIA auf molekulare Teilnehmer der epigenetischen Regulierung untersucht, mit zwei Schwerpunkten - dem Seroton-

intransporter (SERT), da dieser eng mit den pathophysiologischen Prozessen von Depression zusammenhängt, nicht zuletzt durch die bekannte Wirkung von Antidepressiva am SERT; und ausgewählten epigenetischen Markern im Hippokampus des MIA-Nachwuchses. Histon-Acetylierung von H4 im Hippokampus, sowie mRNA Mengen der Histon-Deacetylasen (HDAC) 2 und 9 waren nach MIA reduziert. Diese Gruppe zeigte auch eine Reduktion von SERT mRNA und Protein im Hippokampus. Zusätzlich wurde durch Chromatin-Immunoprezipitation (ChIP) gezeigt, dass die H3 Acetylierung beim SERT Promoter in der MIA-Gruppe reduziert war, während die H4 Acetylierung hier erhöht war. Die Ergebnisse untermauern die Annahme, dass epigenetische Vorgänge die Einwirkung von Umweltfaktoren auf die Entwicklung des Gehirns und das Verhalten durch Veränderungen der Genexpression beeinflussen können. Somit wird vorgeschlagen, dass spezifische Veränderungen in der hippokampalen Histon-Acetylierung - sowohl global als auch Gen-spezifisch - die langanhaltenden Effekte von MIA auf SERT-Expression und Verhalten prägen.

CONTENTS

1. INTRODUCTION

1.1 Background: Major depressive disorder

Major depressive disorder (MDD) is a highly prevalent psychiatric disease, affecting an estimated 10-15% of people worldwide (Bromet et al., 2011). It is characterised by the persistent presence of several emotional, psychological and somatic symptoms including but not limited to depressed mood, anhedonia, fatigue, sleep disturbances, cognitive dysfunctions and suicidal ideation (Holtzheimer and Mayberg, 2011). MDD poses a considerable burden on society as well as on the affected individuals' quality of life, constituting the leading cause of disability worldwide (Whiteford et al., 2013). When examining overall disease burden, which takes into account disability as well as mortality caused by a disease, MDD is the 11th-ranked disease worldwide. However in most developed and high-income countries, MDD has consistently been ranked in the top five with regards to disease burden (Murray et al., 2012). Further, the contribution of MDD to overall disease burden increased by 38% between 1990 and 2010, and this trend can be expected to continue unless significant advances in treatment and disease management are made (Murray et al., 2012).

Akin to other neuropsychiatric diseases, major depression has remained difficult to treat, with several complicating factors intervening in the treatment of MDD patients, including a poorly understood latency in the effect

of antidepressant medication, a high incidence of side effects due to these pharmacological interventions, and the fact that a considerable proportion of patients do not respond to treatments at all (Whiskey and Taylor, 2013; Holtzheimer and Mayberg, 2011). Numerous treatment options exist, both pharmacological and non-pharmacological - a combination of which is usually the most successful in treating the disease (Davidson, 2010).

The dominant pharmacological treatment approaches involve the use of selective serotonin or norepinephrine reuptake inhibitors (SSRIs/SNRIs), tricyclic antidepressants (TCAs) or monoamine oxidase inhibitors (MAOIs) (Davidson, 2010; Levinstein and Samuels, 2014). All of these antidepressants target the molecular machinery of various monoaminergic neurons, thereby exerting significant effects on the neurotransmission in these systems. However the molecular mechanisms of action of these drugs are still not completely understood. An important issue is that most currently prescribed antidepressant drugs show a pronounced latency in their clinical effect that cannot be fully explained by their known activity at the monoaminergic synapse. (Levinstein and Samuels, 2014). Furthermore, treatment is not always successful. Citalopram, one of the most prescribed antidepressants, led to remission in only around 30% of patients in a large multi-step clinical trial (Trivedi et al., 2006). About one-third of MDD patients do not experience remission even after four different established treatments, and as many as 20% of patients do not respond to any available treatments at all, and are thus termed "treatment-resistant" (Rush et al., 2006; Holtzheimer and Mayberg, 2011). Among those who do benefit from the current pharmacological options, a considerable proportion suffer from adverse side effects, which include among others adverse effects on the cardiovascular system, weight gain, nausea, sleep disturbances, and several others that can profoundly affect an individual's

quality of life (Whiskey and Taylor, 2013; Gartlehner et al., 2008). Accordingly, there has been an effort to investigate new pharmacological approaches, one of which includes the use of ketamine, a drug that is widely employed as an anaesthetic: Contrary to traditional antidepressants, this NMDA receptor antagonist has been shown to act within hours of administration, as well as being effective in treatment-resistant patients, though some issues concerning adverse effects remain to be adressed before this drug may enter widespread clinical use (Browne and Lucki, 2013).

Non-pharmacological approaches are also utilised with some success in the treatment of MDD. Different types of psychological interventions, including cognitive behavioural therapy (CBT) and interpersonal psychotherapy, have been shown to be as effective as antidepressant drugs (Picardi and Gaetano, 2014). Furthermore, the combination of psychosocial interventions and pharmacological therapy may offer the most promising treatment outcomes for severe and chronic depression, increasing both recovery rates and decreasing adverse effects (Hollon et al., 2014).

Other non-pharmacological treatments such as electroconvulsive therapy (ECT) have shown considerable efficacy in treating MDD, in fact ECT has been shown to be more effective than some classes of antidepressants, and even works in some patients who were considered "treatment-resistant" (UK ECT Review Group, 2003; Khalid et al., 2008). However, the mechanism of action underlying this technique, which has been in use for over 70 years, is still mostly unknown. A more recent approach paralleling ECT therapy involves the use of deep transcranial magnetic stimulation (deep TMS) for the treatment of major depression, although the initially overwhelmingly positive outcomes remain to be confirmed and some methodological issues are a concern (Morishita et al., 2014).

The issues and unanswered questions described above are further complicated by the heterogeneity and complexity of the disorder, which suggests that many different mechanisms and molecular pathways may contribute to the pathogenesis of MDD. This realisation highlights the need for more individualised treatment plans, which may be more successful in addressing the needs of patients than current therapies (Chien et al., 2013; Davidson, 2010). Further, this approach also emphasises the importance of understanding and therapeutically targeting the molecular disturbances underlying the depressive state.

1.2   Main theories of MDD

One of the first and most prominent theories concerning the aetiology of MDD is the so-called "monoamine hypothesis". This postulates that depression is mainly due to a reduction in monoamine activity in the brain - most importantly in the serotonergic system (Krishnan and Nestler, 2008). This hypothesis is derived mainly from clinical observations and the known mechanisms of action of current antidepressants. However as mentioned earlier, these do not account for some aspects of the clinical effect of these drugs, and the long-term mechanisms involved remain poorly understood (Krishnan and Nestler, 2008). In general, an imbalance in monoaminergic neurotransmission in the brain of depressed individuals is thought to be at the root of the observed alteration in mood and associated symptoms, with antidepressants such as SSRIs targeting these systems to restore the equilibrium (Lee et al., 2010).

Despite the emergence of several other compelling theories in recent years, monoamines - especially the serotonergic system - are still considered fundamental in the aberrant control of mood and mood-related behaviours in

MDD (Haase and Brown, 2014; Lee et al., 2010). The serotonin transporter
(SERT) appears to be particularly important, most likely due to its primary
role in the control of duration and intensity of serotonergic neurotransmis-
sion (Rudnick, 2006; Haase and Brown, 2014). Indeed, SERT polymorphisms
have been among the few identified genetic risk factors contributing to the
development of MDD. Thus, despite remaining controversy, this genetic as-
sociation is widely accepted as being robust, even if the heredity of MDD
remains relatively low at about 40% (Gelernter, 2014; Murphy and Moya,
2011; Sullivan et al., 2000). In fact, in addition to this genetic link and its
obvious involvement in SSRI treatment effects, SERT has been implicated
in MDD pathogenesis by several other lines of evidence, including for exam-
ple a suggested role in BDNF-related hippocampal neurogenesis (reviewed
in Haase and Brown (2014)). Thus SERT appears to play a central role in
the pathophysiological processes involved in depression, and further study of
this transporter is warranted in terms of the monoamine hypothesis, as well
as within the context of other prominent hypotheses concerning MDD.

Further, continued research into the molecular mechanisms associated with
MDD has yielded a variety of other theories concerning the aetiology of this
mental illness, most of which are however beyond the scope of this thesis.
The large number of existing hypotheses - several supported by convincing
evidence - certainly hints at an overarching theme in MDD research: A sin-
gle and unified theory may never be appropriate to describe such a complex
disorder (Krishnan and Nestler, 2008). Indeed, considering the high per-
centage of people diagnosed with depression throughout their lifetime, and
the lack of physiological markers available, it is certainly conceivable that
depression represents a complex of symptoms brought about by distinct sets
of pathophysiological mechanisms rather than representing a distinct disease

that follows a single and definable path. Focusing on these distinct mechanisms which may be at work in different patient subgroups has the potential of leading to more personalised and ultimately more successful treatment strategies.

As such, the following section will introduce a specific theory of MDD that has gained support in recent years, and which led to the rationale underlying this project: the immune theory of depression.

1.3   Inflammation and MDD

In the past few decades, several lines of evidence have provided support for the involvement of the immune system in general, and mediators of inflammation in particular, in the pathophysiology of depression. The following sections will aim to provide an overview of the current opinion and the important studies in this area of research. First, the link between MDD and activated inflammatory pathways and mediators will be summarised. Following this, we will focus on the topic of this project - developmental exposure to immune activation *in utero* as a putative risk factor for the development of depression. Delving first into evidence supporting this rationale from human studies, we will next introduce animal models of maternal immune activation (MIA) and the progress that has been made in the elucidation of the molecular mechanisms of MIA.

1.3.1   MDD and the activation of pro-inflammatory pathways

There have been many studies into the association between MDD and inflammatory states, which was first proposed due to the striking overlap between symptoms shown by depressed patients and so-called "sickness behaviour", which is also characterised by feelings of lethargy, anhedonia and reduced

mood, and which individuals exhibit upon infection with a pathogen (Dantzer et al., 2008). Sickness behaviour has been proposed to be caused mainly by the action of particular pro-inflammatory cytokines, which despite their initially protective function can exert maladaptive effects if the inflammatory state is prolonged - the most relevant here being interleukin-1β (IL-1β), IL-6 and tumor necrosis factor α (TNF-α) (Dantzer et al., 2008).

Several meta-analyses have reported an association between elevated plasma levels of the pro-inflammatory cytokine IL-6 and major depression, providing strong support for its involvement - direct or indirect - in MDD pathogenesis. (Dowlati et al., 2010; Valkanova et al., 2013; Howren et al., 2009). Dowlati et al. (2010) also examined a range of other inflammatory markers and found another robust association of MDD diagnosis with increased levels of TNF-α, however the often-reported increase in IL-1β (as well as changes in the levels of other cytokines) did not show a statistical association with MDD in this meta-analysis.

Further, acute administration of a low-dose endotoxin (*Salmonella abortus equi*) that induces cytokine release - including that of TNF-α and IL-6 - was shown to induce transient anxiety and depression as well as cognitive deficits in healthy adults, with a correlation between severity of mood change and cytokine levels apparent in the tested individuals (Reichenberg et al., 2001).

Thus while the evidence for an involvement of inflammatory mediators in MDD is strong, whether cytokines intervene in the causative mechanisms of depression or whether MDD causes disturbances in the immune system leading to imbalance of cytokine levels needs to be addressed in further studies, and it is also conceivable that both depression and immune activation result from a third mechanism that has not yet been identified (Hannestad

et al., 2011). Furthermore, it should be noted that it is still not entirely clear how inflammatory mechanisms in the periphery - which are often focused on - mediate changes that are thought to occur in the central nervous system, especially in light of the immunological separation of these two compartments (Miller et al., 2009). Though several theories exist, including for instance the dysregulation of neuroendocrine systems due to peripheral inflammation, which will be briefly discussed below, research needs to continue in this area to confirm the causal mechanisms involved (Anisman and Merali, 2003; Raison and Miller, 2011).

In search of alternative pharmacological treatment options for MDD, researchers have turned to evaluating the effects of anti-inflammatory drugs, some of which are already in use for treating other conditions. This would greatly reduce the extent and length of clinical trials necessary to bring them into standard clinical practice for MDD treatment, a factor that should not be underestimated in drug development - both in terms of time considerations and economic costs.

Celecoxib, a non-steroidal anti-inflammatory drug (NSAID), was demonstrated to have positive effects on both response and remission rates when used as an add-on therapy to sertraline in the treatment of MDD patients, and the treatment outcome was significantly correlated with reductions in plasma levels of IL-6 observed (Abbasi et al., 2012). Impressively, the response rate to the treatment was almost doubled to 95% with additional celecoxib (Abbasi et al., 2012). Previously, similar studies had shown that celecoxib also significantly improves treatment outcome when used in addition to reboxetine (Müller et al., 2006) and fluoxetine (Akhondzadeh et al., 2009), and a meta-analysis found this increase in treatment efficacy to be a robust effect (Na et al., 2014), although more studies are necessary to confirm

these promising results.

In contrast, in a small clinical trial that aimed to examine the utility of aspirin as an adjunctive therapy to citalopram (SSRI), severe adverse reactions were observed and medication had to be discontinued in most cases (Ghanizadeh and Hedayati, 2014), which shows that only some classes of anti-inflammatory drugs may be suited for combination therapy with SSRIs when treating MDD.

While the mechanisms underlying the contribution of inflammatory mechanisms to MDD remain poorly understood, there are theories relating to the mechanisms at hand, which have been readily integrated into more established hypotheses, creating an ever more complex and intricate picture of this common mental illness.

Briefly, it is thought that the cytokines that have been observed in association with MDD are produced by activated T lymphocytes and monocytes, which are important immune cells (reviewed in Maes (2011)). Some cytokines are thought to induce the activity of indoleamine 2,3-dioxygenase (IDO), which catabolises the reaction of tryptophan - the precursor of serotonin - into tryptophan catabolites (TRYCATs, e.g. kynurenic acid), effectively diverting the use of tryptophan away from the serotonin-producing pathway (Maes, 2011; Maes et al., 2011). On one hand, this is thought to reduce levels of serotonin globally, which would tie in with the monoamine hypothesis of MDD, while on the other hand some of the TRYCATs are thought to possess neurotoxic, depressogenic and anxiogenic effects (Wichers et al., 2005; Maes, 2011; Maes et al., 2011). Further, it is thought that the induced pathways may lead to decreased neurogenesis, which has been shown to be important in MDD (Anderson et al., 2013). Thus this hypothesis of depression postulates that pro-inflammatory cytokines lead to a decrease in serotonin synthesis while

promoting an increased production of potentially detrimental TRYCATs, thereby contributing to the development of MDD. However, it is still unclear why the immune system becomes activated to the point of inducing such maladaptive processes, and this question certainly warrants further research.

In conclusion, there is strong support for an immune theory of MDD from several lines of research, though there are still unanswered questions concerning the nature of the molecular mechanisms that may underlie any potential causative links. While the evidence supports a role for general inflammatory pathways in the development of depression and its treatment, it has also been suggested that they can have a powerful impact on prenatal development when the brain is exposed to the mother's immune response against a pathogen. This developmental effect, also purported to be linked to major depression, will be further investigated in this research project. An outline of the relevant research concerning maternal immune activation (MIA) and its effect on the offspring, both in humans and animal models, will be given in the following sections.

1.3.2 Evidence for a link between MDD and MIA

The association between major depression, other neuropsychiatric disorders and elevated activity of the immune system during pregnancy has been observed for years, in part due to the noteworthy link between the season of birth and an increase in psychiatric diagnoses: Affective disorders were increased in individuals born during the spring and early summer months, a finding that initiated the hypothesis proposing that an increased risk of bacterial or viral infection to pregnant women during winter months may influence the risk for future mental health issues in the offspring (Castrogiovanni et al., 1998; Fountoulakis et al., 2007). Indeed, further epidemiological stud-

10

ies have confirmed that exposure to MIA due to an infection of the mother by a pathogen is associated with the development of psychiatric disorders in the adult offspring (Brown, 2012).

Generally, it is widely accepted that maternal infection during pregnancy can contribute to the development of schizophrenia of the offspring in later life (Meyer and Feldon, 2009). On the other hand, affective disorders such as MDD have also been linked to prenatal infection, though the studies are not as numerous or as consistent as with schizophrenia. Table 1 summarises the results of epidemiological studies that have been undertaken to investigate the link between MIA and neuropsychiatric disorders. The link between schizophrenia and prenatal immune stimulation by a pathogen is thus supported by a large number of studies, although evidence to the contrary exists as well (Table 1). In general, the studies concerning a link between MIA and depression in later life are much sparser than in the case of schizophrenia, and present with contradictory results (Table 1). Considering the convincing link between inflammatory mechanisms and the development of MDD discussed above, further investigation into the link between prenatal infection and depressive disorders is certainly warranted despite the current lack of consensus.

The mechanism by which maternal infection affects the foetus is highly complex, in part due to the fact that the maternal immune system must inhibit a rejection of the foetus itself but must at the same time maintain vigilance against possible infectious pathogens.

At the interface between the mother and the foetus lies the placenta, which expresses many types of Toll-like receptors (TLRs) - part of the innate immune system, these receptors recognise pathogen-associated molecular patterns (PAMPs) and lead to activation of signalling cascades that result in

11

Disorder	Reference	Pathogen	Results	Effect on risk for disorder
affective disorders	Brown et al. (1995)	influenza virus	reduced risk of developing affective disorders after putative exposure to influenza epidemic	⇓
major depression	Machón et al. (1997)	influenza virus	increase in major depression diagnoses after putative exposure to influenza epidemic	⇑
major depression	Mino et al. (2000)	influenza virus	no effect found	⇔
major depression	Pang et al. (2009)	various viral agents (total effect analysed)	no effect found	⇔
schizophrenia/ affective psychotic disorders	Takei et al. (1993)	influenza virus	increased schizophrenia diagnoses after putative exposure to influenza epidemic; decrease in affective psychotic disorder diagnoses	⇑ (SCZ), ⇓ (affective psychotic disorder)
schizophrenia and depressive illness	Cannon et al. (1996)	influenza virus	increased risk for depressive illness among exposed individuals; no significant effect on schizophrenia diagnoses found	⇑ (MDD), ⇔ (SCZ)
schizophrenia and affective psychosis	Cahill et al. (2002)	poliovirus	no effect found	⇔
schizophrenia	Watson et al. (1984)	various infectious diseases	increased risk for schizophrenia in individuals with birth years directly following time periods of high prevalence of infectious diseases	⇑
schizophrenia	Mednick et al. (1988)	influenza virus	increased risk for schizophrenia in individuals exposed to influenza during gestation	⇑
schizophrenia	Torrey et al. (1988)	various (total effect analysed)	increased risk for schizophrenia after putative exposure to measles (m), varicella zoster (vz), polio (p); influenza showed effect just below significance level; no effect of rubella (r) or mumps (mu) found.	⇑ (m, vz, p) ⇔ (r, mu)
schizophrenia	Barr et al. (1990)	influenza virus	increased schizophrenia diagnoses in individuals exposed to periods of high incidence of influenza during gestation	⇑
schizophrenia	O'Callaghan et al. (1994)	influenza virus	increase in births of schizophrenic individuals 5 months after peak infection prevalence	⇑
schizophrenia	Crow and Done (1992)	influenza virus	no effect found	⇓

Table 1. Epidemiological evidence for a link between prenatal infection and subsequent development of different neuropsychiatric disorders (continued on next page).

Disorder	Reference	Pathogen	Results	Effect on risk for disorder
schizophrenia	Adams et al. (1993)	influenza virus	increased schizophrenia diagnoses after influenza epidemics in three cohorts of patients	⇑
schizophrenia	Susser et al. (1994)	influenza virus	no effect found	⇓
schizophrenia	Suvisaari et al. (1999)	poliovirus	increased schizophrenia diagnoses in individuals exposed to periods of high incidence of poliomyelitis during gestation	⇑
psychotic disorders	Brown et al. (2000)	rubella virus	increased risk for nonaffective psychosis among individuals exposed to rubella during gestation	⇑
psychotic disorders	Buka et al. (2001)	analysis of antibodies in maternal serum at birth	association between elevated maternal levels of IgG, IgM and antibodies against HSV-2 (but not against several other pathogens) and schizophrenia diagnoses in offspring	⇑ (HSV-2)
schizophrenia	Limosin et al. (2003)	influenza virus	increased risk for schizophrenia in individuals exposed to influenza during gestation	⇑
schizophrenia	Brown et al. (2004)	influenza virus	increased risk for schizophrenia in individuals exposed to influenza during gestation	⇑
schizophrenia	Brown et al. (2005)	Toxoplasma gondii	increased risk for schizophrenia spectrum disorders in subjects with high maternal levels of T. gondii antibody	⇑
schizophrenia	Babulas et al. (2006)	maternal genital/reproductive infections	increased risk for schizophrenia and schizophrenia spectrum disorders in individuals exposed during the periconceptional period	⇑
schizophrenia	Mortensen et al. (2007)	Toxoplasma gondii	increased risk for schizophrenia in subjects with high maternal levels of T. gondii IgG antibody	⇑
psychotic disorders	Buka et al. (2008)	herpes simplex virus	increased risk of developing psychoses in offspring of mothers seropositive for herpes simplex	⇑
schizophrenia	Sørensen et al. (2009)	various bacterial agents (individual/grouped effects analysed)	increased risk for schizophrenia in individuals exposed to various bacterial pathogens during gestation	⇑
schizophrenia	Ellman et al. (2010)	IL-8	increased risk for presenting neuroanatomical changes that have been previously linked to schizophrenia among cases exposed to IL-8 in utero	⇑

Table 1 (continued). Epidemiological evidence for a link between prenatal infection and subsequent development of different neuropsychiatric disorders.

the production of pro-inflammatory molecules, including cytokines (Riley and Nelson, 2010). During normal development, the balance of cytokines between maternal and foetal compartments is tightly regulated as in addition to their role in immunity, cytokines also function as signalling molecules to direct developmental processes (Garay et al., 2013; Deverman and Patterson, 2009). Contrary to most pathogens (Robbins and Bakardjiev, 2012), cytokines can cross the placental barrier and even the blood-brain-barrier (Patterson, 2007; Meyer et al., 2006). Thus maternal infection may lead to an invasion of the foetal compartment as well as the foetus itself by maternal cytokines, which may interfere with correct development - including that of the brain, which may explain the association between MIA and neuropsychiatric disorders.

Nevertheless, by their observational nature, most of the studies investigating a link between MDD and MIA in human populations have not been able to further our understanding of the molecular mechanisms underlying this disturbance of foetal brain development by MIA. Therefore, animal models of MIA have been invaluable tools in the study of these causative links due to the potential for experimental manipulation. Several animal models of MIA exist, most of which involve the administration, during gestation, of live pathogens or substances which elicit an immunological response comparable to a viral or bacterial infection. In particular, the use of the bacterial cell wall component lipopolysaccharide (LPS) and the viral mimetic Poly(I:C) are widespread (Meyer et al., 2009; Meyer, 2014). However, the focus of this project is the Poly(I:C) model of MIA, which will be the topic of the next section.

1.3.3   Investigation of the mechanisms underlying the link between MIA and MDD

Several animal models of MIA are in use today, the most popular entailing the administration during gestation of either LPS or Poly(I:C) (Polyinosinic:polycytidylic acid) (Meyer et al., 2009), the latter of which will be the focus of this project. In the Poly(I:C) model of MIA, Poly(I:C) is administered to pregnant rodents to mimic a viral infection. The offspring can then be investigated to elucidate potential mechanisms that underlie the detrimental effects of MIA (Meyer et al., 2009). Poly(I:C) is a synthetic analogue of double-stranded RNA (dsRNA) and thus constitutes a viral mimetic (Tatematsu et al., 2014). Under normal circumstances, dsRNA is only present in an organism when it is under attack by a pathogen: Viral dsRNA may constitute the viral genome itself or may arise from replication of RNA or DNA viruses within the host organism; and some studies have shown that endogenous dsRNA can also be released from necrotic cells (Tatematsu et al., 2014). Both endogenous and exogenous dsRNA, as well as Poly(I:C), can activate Toll-like receptor (TLR) 3 - which is considered to be essential in the innate immune response against most viruses (Perales-Linares and Navas-Martin, 2013). TLR3 is expressed in the endosomes of immune cells as well as non-immune cells such as neurons (Zhang et al., 2013). As shown in Figure 1, the activation of TLR3 leads to signal transduction cascades that promote the upregulation of anti-viral, pro-inflammatory and pro-apoptotic factors (Riley and Nelson, 2010; Perales-Linares and Navas-Martin, 2013). These immune responses to viral pathogens include the production of pro-inflammatory cytokines which may be detrimental to the health of the developing foetus when exposed *in utero* (Garay et al., 2013; Deverman and Patterson, 2009). Several molecules of the TLR3 pathway have been recently implicated in the

15

aetiology of neuropsychiatric disorders, which lends further support to the use of a TLR3 agonist as an investigative tool in this line of research. Very briefly, it has been shown that schizophrenia is associated with abnormal expression of the TLR3 receptor (Figure 1/ label 1) (Müller et al., 2012; Chang et al., 2011). Similarly, increased levels of TLR3 in the brain have been found to be associated with suicide among depressed individuals as well as among other neuropsychiatric patients (Hoyo-Becerra et al., 2013; Pandey et al., 2014).

Suggesting its involvement in pathogenic mechanisms of another neuropsychiatric disorder, SARM1 (sterile alpha and TIR motif containing 1, Figure 1/ label 2) knockdown in mice impaired synaptic function and led to behavioural abnormalities usually associated with autism spectrum disorders – and the SARM gene is also part of an autism susceptibility locus (*Auts6*), highlighting the relevance of this finding (Lin and Hsueh, 2014; Lin et al., 2014).

Moreover, a study showed that as well as being increased in depressed patients pre-treatment, levels of NF-kB (nuclear factors kappa-B, Figure 1/ label 3), also induced as part of the TLR3 pathway, dropped after cognitive behavioural therapy, inversely correlating with clinical improvement experienced by patients (Kéri et al., 2014). The same study showed that the cytokine levels (Figure 1/ label 4) of depressed patients undergoing cognitive behavioural therapy also dropped significantly during the treatment, with the magnitude of the decrease appeared to correlate with the degree of improvement experiences due to CBT, supporting the involvement of NF-kB in either MDD or the therapeutic effect of CBT (Kéri et al., 2014).

In addition to the associations discussed previously, it was found that in
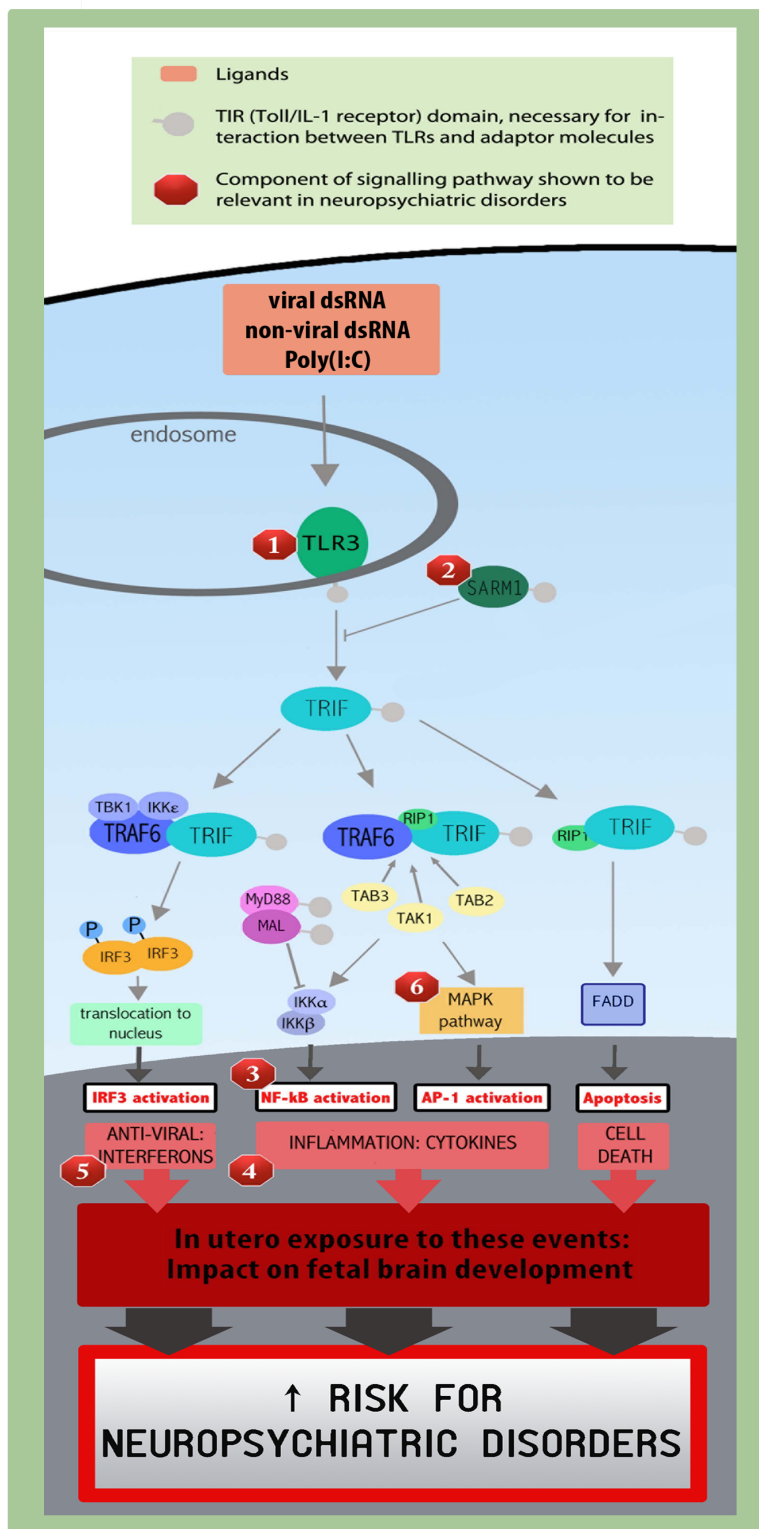
Figure 1. Involvement of the TLR3 pathway in maternal immune activation and its link to neuropsychiatric disorders.
Several components of the TLR3 signal transduction pathway have been linked to mechanisms underlying neuropsychiatric disorders, illustrating the validity of using this pathway in the study of MIA and its effects on mental health.

Abbreviations: AP-1 (activator protein 1), FADD (=Fas-associated protein with death domain), IKKs (=inhibitors of NF-kB kinase), IRAKs (=interleukin-1 receptor-associated kinase) 1 and 4, IRF3 (=Interferon regulatory factor 3), MAL (=MyD88 adaptor-like), MyD88 (=Myeloid differentiation primary response gene 88), NF-kB (=nuclear factor kappa-light-chain-enhancer of activated B cells), SARM1 (=Sterile alpha and TIR motif containing 1), TAK1 (=Transforming growth factor beta activated kinase-1), TRAF3 (=TNF receptor-associated factor 3), TRAF6/RIP1 (=TNF receptor-associated factor 6/receptor interacting protein 1), TRAM (=TRIF-related adaptor molecule), TRIF (=TIR-domain-containing adapter-inducing interferon-β).

schizophrenic patients and bipolar patients, exposure to TLR agonists led to a significantly higher cytokine release in the psychotic patients than in the healthy group (McKernan et al., 2011). Another study found that IL-6 and IL-10 mRNA levels were significantly higher in monocytes of individuals with schizophrenia compared to healthy controls, supporting the notion of a hyperactive immune system in these patients (Chang et al., 2011). These findings suggest an altered sensitivity to pathogens in psychiatric patients, which may well result in increased detrimental effects from cytokine release. Furthermore, administration of the cytokine IFN-α (Figure 1/ label 5) in Hepatitis C therapy leads to severe depression in a large proportion of patients (Loftis and Hauser, 2004). A set of genes shown to be upregulated in postmortem samples of suicidal depressive patients and the blood of depressive patients was also shown to be upregulated upon simultaneous in vitro TLR3 stimulation with Poly(I:C) and murine IFN-α, providing insight into the potential mechanisms underlying IFN-α-induced depression - and therefore MDD (Schlaak et al., 2012; Hoyo-Becerra et al., 2013).

Finally, disturbances of the MAPK pathway (Figure 1/ label 6) in mice have been linked to increased anxiety-related behaviour and depression-related behaviour, depending on whether the gene inactivation took place during the juvenile phase or during adulthood (Wefers et al., 2012), suggesting that this molecular pathway may intervene in processes that are relevant to MDD and anxiety.

Accordingly, MDD and neuropsychiatric disorders in general have been repeatedly associated with disturbances in the TLR3-mediated signalling pathway, highlighting the validity of the Poly(I:C) model for use in neuropsychiatric research. It thus appears that the response initiated by TLR3 is initially adaptive in that it contributes to the fight against a pathogen, yet the ac-

tivated downstream effectors including cytokines and interferons may cause damage to the host organism - which is all the more consequential if exposure to such effectors occurs during the sensitive period of neural development (Perales-Linares and Navas-Martin, 2013).

When using animal models of neuropsychiatric diseases, it is important to keep in mind the limitations of such models when interpreting results. While this is true to some extent for all animal models of human disease, mental illnesses may appear especially unsuited due to their complexity and the seemingly uniquely human aspects of some of the symptoms, such as suicidal ideation, delusions or psychotic episodes. Therefore, it is especially crucial to refrain from anthropomorphising the animals and their behaviours, instead focusing on the modelling of the underlying molecular mechanisms, which may be observed thanks to mental illness-*related* phenotypes such as behaviour and endophenotypes such as alterations to relevant neurotransmitter systems, brain structures, or the expression of relevant genes (Hall et al., 2014). Importantly, it is futile to attempt to model the entirety of a complex neuropsychiatric disorder in animals such as rodents, instead the focus needs to be on the modelling of particular pathways or disease processes (Meyer and Feldon, 2012). If keeping this rationale in mind, however, animal models provide an invaluable tool to study causative mechanisms underlying neuropsychiatric diseases as they allow experimental manipulations that are not feasible in human studies, which often must rely on observation of symptoms or imaging techniques only, while molecular studies of the brain tissue may only take place postmortem (Pollak et al., 2010; Zipursky, 2007).

One proposed limitation of investigating animal models of MIA specifically is that they do not exhibit the full range of immune responses to pathogens - however they do replicate the cytokine-mediated acute phase of the response

to bacterial or viral infections (Meyer et al., 2009). It is argued that the Poly(I:C) model for MIA is therefore aptly suited to replicate the effects of the maternal, virus-induced cytokine response on the developing foetus, which is thought to constitute one of the essential pathogenic mechanisms underlying MIA (Meyer et al., 2009). Moreover, a manipulation during development, such as the administration of Poly(I:C), bears a distinct advantage in modelling the actual disease aetiology over animal models in which the experimental manipulation takes place in the adult organism, since in humans it is thought that environmental insults during development play a fundamental role in the advent of psychiatric disorders, effects which may not be recapitulated in non-developmental models (Powell, 2010; Meyer et al., 2009).

The approach using animal models of MIA therefore promises to yield insight into a complex set of potentially interacting molecular mechanisms pertaining to aberrant development of the brain, which needs to be elucidated to explain the causative mechanisms at hand - especially in view of intriguing epidemiological findings, which unfortunately cannot offer any insight into causal links. As such, animal models - despite their drawbacks - remain extremely valuable tools in neuropsychiatric research simply because they allow the direct testing of hypotheses concerning the neurobiological underpinnings of disease aetiology (Reisinger et al., 2015).

Although the Poly(I:C) model is established in the investigation of an association between MIA and schizophrenia (Meyer, 2014), more recently it has also been used to examine the effect of MIA on behaviours and molecular mechanisms related to MDD . For instance, a study from this laboratory investigated the effect of Poly(I:C) treatment on a variety of behaviours and molecular processes related to MDD: It was demonstrated that adult MIA-

exposed mice showed a significant increase in depression-like behaviours as measured by the sucrose preference and forced swim tests, two established behavioural paradigms in the field of MDD research, while general behaviour such as locomotor activity were not affected (Khan et al., 2014). Cognitive deficits were also observed in these mice, and these were accompanied by impairments in hippocampal long-term potentiation (LTP) and paired-pulse facilitation - processes indicative of pre- and post-synaptic function respectively (Khan et al., 2014). This was further paralelled by a reduction in hippocampal neurogenesis, and changes in the expression of VEGFA (vascular endothelial growth factor A) and its receptor VEGFR2, all of which have been previously shown to be relevant in MDD (Khan et al., 2014; Lee and Kim, 2012). These results show that MIA leads to altered behaviours and cellular dysfunctions that are relevant to MDD, illustrating that MIA may represent an environmental risk factor that intervenes in normal neural development - with long-lasting consequences - potentially causing deficits in the adult offspring that may manifest as MDD. Moreover, these results provide support for the use of the Poly(I:C) model in MDD research.

Considering the existing overlap in symptoms between several psychiatric conditions, and as mentioned above in relation to the limitations of animal models, it is worth noting that the Poly(I:C) model may be more useful to model certain aspects of a disease - in this case affective-, social- and anxiety-related symptoms of psychiatric conditions - rather than a recapitulation of a single psychiatric disorder. Bearing this in mind, it no longer appears surprising that the same animal model can be used to investigate various different neuropsychiatric disorders.

In conclusion, the studies undertaken to date using the Poly(I:C) model of MIA broadly support its relevance for the study of neuropsychiatric dis-

orders, and in addition to its already established application as a model for MIA-induced schizophrenia, it has proved itself to be a pertinent tool for the study of other disorders - including MDD. In this project, one of our aims was therefore to investigate the molecular mechanisms underlying the described alterations in offspring behaviour and gene expression relevant to MDD. Thus the goal was to examine how an early environmental insult may impact the organism. With regard to this, epigenetic regulation of gene expression is widely thought to represent a potential molecular substrate of the interaction between the environment and our genes, and thus constitutes a fitting area of study to elucidate the effects of MIA on traits and genes relevant to MDD. The following section will provide an overview of relevant epigenetic mechanisms and introduce important findings concerning epigenetic regulation with regards to the pathogenesis of MDD.

1.4   Epigenetics and MDD

1.4.1   Epigenetic regulation of gene expression: background and relevance for complex disorders

The expression of genes needs to be highly controlled - both in terms of cellular location and developmental time point. This allows the organism to ensure that each cell is expressing only genes that it needs, thus not expending any unnecessary energy on the production and degradation of incorrectly synthesised molecules (Shahbazian and Grunstein, 2007). Considering this obvious need, it became clear even before the discovery of DNA that a regulatory system must be in place to ensure that only necessary genes are transcribed in a given cell, and that this must take place without altering the genes themselves - these mechanisms were collectively termed "epige-

netics", and this insight marked the start of a new and still ever-expanding field of biology (Waddington, 1939; Portela and Esteller, 2010). Although epigenetic mechanisms are considered critical for most important physiological and pathological processes, their relevance is proposed to be especially poignant in the case of complex disorders: Here, it is thought that a dysregulation of epigenetic mechanisms may account for the interaction between genes and environmental factors that appears to underlie the development of such conditions (Portela and Esteller, 2010). Epigenetic regulatory mechanisms, which work without any modification of the genetic sequence itself - instead relying on diverse modifications to the DNA molecule or to the histones that make up nucleosomes, around which the DNA is wrapped to create highly compacted chromatin - may represent the biological foundation of this *Gene × Environment* interaction in complex disorders (Lee and Lee, 2012; Petronis, 2010). Strictly speaking, environmental factors such as MIA can only have a direct influence on the genes - specifically the DNA sequence - within the cell where a mutation occured. In contrast, the molecular mechanisms by which genes are regulated, as part of the cellular machinery, may be manipulated directly by environmental factors - potentially leading to long-lasting and wide-spread changes in expression, which can also be transmitted to daughter cells - thereby constituting a legitimate mechanistic link between the concepts of *Nature* and *Nurture* (Petronis, 2010). In particular, several aspects of psychiatric disorders fit in well with this concept, for instance the observed discordance of mental illness diagnoses between monozygotic twins - accompanied by a relatively weak genetic link, late age of onset, a variability in disease course (Ptak and Petronis, 2010; Mill and Petronis, 2007). Further, it is important to note that because neurons are post-mitotic and therefore do not divide, alterations to chromatin structure and epigenetic

23

mechanisms are maintained within the neurons and are not lost as readily as in other more proliferative tissues, potentially making these changes especially relevant in neurological and neuropsychiatric disorders (Tsankova et al., 2007).

As such, it has emerged in recent years that alterations in epigenetic regulation - permanent or transient - may represent a unifying, fundamental principle of complex diseases, which cannot be explained by Mendelian inheritance or environmental influences alone (Petronis, 2010).

Therefore, the study of epigenetic mechanisms and their dysregulation may yield insight into the pathological mechanisms at work in many complex diseases, and additionally may provide new targets for pharmacological therapy. Indeed, the plethora of molecules intervening in epigenetic regulation may allow highly targeted therapies if the dysfunctional mechanism that underlies a disorder can be identified with precision.

Several processes are thought to be of crucial importance in the epigenetic regulation of gene expression, a short outline of which will be provided here. Chromatin is the tightly packaged form of DNA, which enables the entire genome of an organism to fit into the nucleus of a cell - all achieved by the DNA being wound around proteins termed nucleosomes, which are themselves composed of histone octamers (Rothbart and Strahl, 2014). Generally, the structure of chromatin inhibits access of the transcriptional molecular machinery to the DNA molecule, thereby limiting transcriptional activation unless it is necessary - hence the structure must not only be dynamic but also tightly and precisely regulated (Swygert and Peterson, 2014). This regulation is thought to be achieved by several processes, which can be divided into three main categories. However, as the field is constantly evolving, new epigenetic mechanisms may be identified in the future.

Firstly, genes and their expression may be regulated by methylation of the DNA in regions termed "CpG islands" - clusters of cytosine/guanosine dinucleotides - which is thought to have a repressive effect on gene expression via several possible mechanisms, and which will not be elaborated upon here (reviewed in Portela and Esteller (2010)).

Further, modifications to the nucleosomes, around which the DNA is wrapped when it is in the chromatin state inside the nucleus of a cell, are thought to be important: Histones, the proteins that compose nucleosomes by forming octamers between different types (including H2A, H2B, H3 and H4 histones), may be acetylated, phosphorylated, methylated, sumoylated (and more), leading to a variety of effects on transcription (Rothbart and Strahl, 2014). In particular, histone acetylation is widely believed to be related to an increase in gene expression (Portela and Esteller, 2010; Shahbazian and Grunstein, 2007), though this view has been contested by some (Henikoff and Shilatifard, 2011; Ptashne, 2013). Due to the purported importance of histone acetylation in the regulation of gene expression, the proteins that acetylate and deacetylate histones - HATs (histone acetyl-transferases) and HDACs (histone deacetylases) - have also received much attention from researchers looking into epigenetic regulation, both in terms of their basic function in the healthy organism and in terms of potential drug targets (Lombardi et al., 2011; Schneider et al., 2013). Importantly, HATs and HDACs act in a targeted manner, recruited by transcriptional activators and repressors at particular loci, as well as globally, and gene expression seems to be influenced by both local and global levels of acetylation (Shahbazian and Grunstein, 2007).

While both DNA and histone modifications are thought to at least in part exert their effects by influencing the structure of chromatin (Lee and Lee, 2012),

external mechanisms may also intervene: The third fundamental mechanism of epigenetic regulation is thought to entail structural reorganisation of the chromatin by a variety of so-called chromatin-remodelling enzymes, which are thought to act by binding to histone modification motifs, changing the conformation of the chromatin - thereby allowing more or less access to a particular DNA sequence for the transcriptional machinery (Swygert and Peterson, 2014).

Thus several epigenetic mechanisms that contribute to the correct regulation of gene expression have been identified and studied in detail. However complex these mechanisms may seem, they are often interpreted in a relatively simplistic manner, an approach that is being challenged lately as an even more complex picture is emerging: Interactions between different histone modifications as well as with DNA modifications and the chromatin-remodelling enzymes, in addition to differential effects of histone modifications depending on their location, have been reported - though the details of these processes remain to be elucidated (Turner, 2014; Swygert and Peterson, 2014; Ptashne, 2013; Henikoff and Shilatifard, 2011).

As a complex and multifactorial disorder, the study of epigenetic control thus appears as a sensible approach to investigate MDD in general, and MIA as a risk factor in particular, for probing the disease mechanisms as well as finding new pharmacological targets for treatment of depression. In the next section, recent relevant findings concerning the epigenetics of MDD will be introduced.

1.4.2   Epigenetic processes and the pathophysiology of MDD

The study of epigenetic mechanisms and their dysregulation in in the context of MDD has emerged as a promising approach in the clarification of the

underlying pathogenic mechanisms as well as the search for new therapeutic options. For brevity, only most important and relevant findings will be presented here.

For instance, several studies have found an association between depression-like behaviour in animal models and an increase in methylation at the BDNF (brain-derived neurotrophic factor) promoter, a gene that has been shown to be involved in depression as well as antidepressant activity (Tsankova et al., 2006; Kundakovic et al., 2014). Interestingly, this observed increase in histone methylation at BDNF has since been confirmed in several studies of human MDD patients, indicating that this histone marker may be clinically relevant as well as potentially useful as a biomarker for MDD (Fuchikami et al., 2011; Song et al., 2014; Carlberg et al., 2014).

Further, in a rodent model of MDD, it has been shown that depression-related traits are accompanied by alterations in the levels of acetylation in the hippocampus (Covington et al., 2011) and the nucleus accumbens, the latter of which were also observed in the postmortem brain tissue of MDD patients (Covington et al., 2009). Finally, it has been reported that changes in levels of HDACs in several brain regions are associated with depression-like behaviour (Covington et al., 2009; Réus et al., 2013) as well as the effect of specific antidepressants (Tsankova et al., 2006; Covington et al., 2011). Intriguingly, the administration of an HDAC inhibitor partially blocked the behavioural effect of the social defeat paradigm, a behavioural paradigm that induces depression-like behaviour, which suggests that HDACs could potentially represent viable targets for pharmacological therapy, as well as suggesting their involvement in the aetiology of MDD (Covington et al., 2011).

While this short introduction to the research concerning epigenetic mechanisms associated with MDD is far from exhaustive, it gives an indication of

the potential of this area of research in furthering our understanding of this prevalent neuropsychiatric disorder. Both in terms of clarifying the nature of the impact of environmental influences on the development of MDD as well as providing new drug targets for therapy, this avenue of research promises to yield valuable new insights in the future.

1.5   Rationale, aims and objectives

Although the impact of infectious stress during gestation as a critical early life insult impacting on the development of depressive-like behaviour has been demonstrated, the role of epigenetics as the modulatory interface driving the underlying gene expressional and behavioural alterations has not yet been studied.

Aiming to investigate the long-lasting effects of MIA on specific aspects of the epigenetic machinery in the adult offspring hippocampus, a brain region highly implicated in the aetiology of MDD (Posener et al., 2003; McKinnon et al., 2009), with specific consideration of the serotonin transporter, we here used the Poly(I:C) mouse model of gestational infection to experimentally test three basic hypotheses:

H1. MIA induces persistent changes in hippocampal SERT expression.

H2. MIA leads to a global modulation of the epigenetic profile in the adult offspring hippocampus.

H3. Histone modifications at the promoter region drive life-long alterations in hippocampal SERT expression resulting from MIA.

An outline of the experimental design can be found in Figure 2.

Accordingly, we pursued the following specific aims:

A1. To quantify SERT expression at the mRNA and protein level in hippocampal tissue of adult MIA and control offspring.

A2. To examine hippocampal content of total and acetylated H3 and H4 protein in adult MIA and control offspring and to determine mRNA levels of the regulatory enzymes HDAC 1 through 11.

A3. To determine the acetylation status of H3 and H4 at the SERT promoter in adult MIA and control offspring hippocampus.

As such, the overall aim of this project was to provide a mechanistic link between maternal infection as an environmental insult that may increase the risk of developing MDD, and epigenetic alterations observed at the molecular level with a special focus on SERT - a protein particularly implicated in the pathophysiology of MDD and its pharmacological treatment.
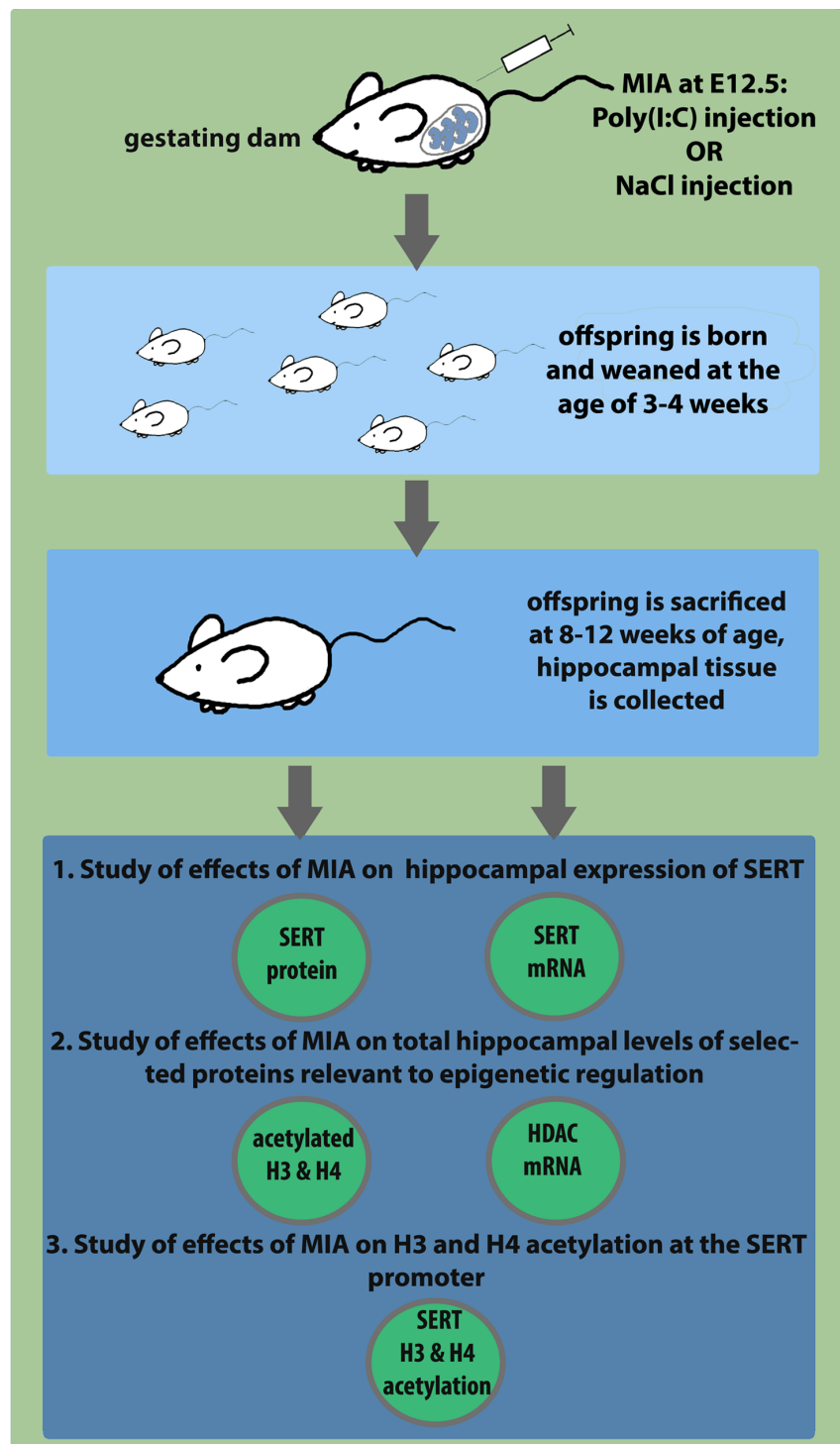
Figure 2. Experimental outline of research project. The Poly(I:C) rodent model was used to investigate the effects of MIA on SERT expression and epigenetic markers in the adult hippocampus.

2. MATERIALS AND METHODS

2.1 Animals

2.1.1 General

All animals used for this project were C57BL/6N mice obtained from Charles River (Sulzfeld, Germany).

Animal experiments described in this study were approved by the national ethical committee on animal care and use in Austria (Bundesministerium für Wissenschaft und Forschung: BMWF-66.009/0015-II/3b/2012) and were carried out according to international laws and policies.

2.1.2 Timed mating

Female mice were group housed for up to a week prior to mating to promote the synchronisation of the females' oestrous cycle according to the Lee-Boot effect (McClintock, 1984). To further increase the chances of successful conception upon meeting of the mating pairs, the females were then co-housed with male mice for two days (48 hours) prior to mating. This technique takes advantage of the Whitten effect, by which exposure to olfactory cues from a male greatly increases the chances of oestrus onset and thus heightens the probability of subsequent conception (Gangrade and Dominic, 1984). This was achieved using large cages equipped with a perforated Plexiglas wall separating two sections of the cage to limit physical contact between male and

female mice, while still allowing olfactory communication and the passage of airborne pheromones. In addition, soiled bedding from male mice was put into the side occupied by the females to enhance their exposure to male pheromones. Three to four females were placed on one side of the partition, with one male occupying the other side.

Following 48 hours of co-housing, each female was placed into the home cage of a male overnight (12 hours) for mating. This ensured that the time point of conception, embryonic day 0 (E0), could be pin-pointed with an accuracy of 12 hours, which is important for the timing of the ensuing maternal immune activation by Poly(I:C), which was carried out at E12.5.

After the end of the mating period, male mice were removed and females were left single housed. The presence or absence of vaginal plugs in the females was recorded: Vaginal plugs are composed of hardened constituents of semen and are intended to prevent another male from successfully mating with the female, and are thus often observed when copulation has taken place, although additional measures such as weight gain may be preferable for assessing pregnancy with certainty (Mader et al., 2009). The females were weighed at E0 and several times throughout their pregnancy until E12.5.

2.1.3 Administration of Poly(I:C)

At the time point E12.5 the total weight gain (%) since mating was calculated for each dam. C57BL/6 mice can be expected to gain 25%-40% of their initial body weight during the first 12.5 days of pregnancy, allowing the determination of pregnancy with up to 99 % certainty at this time point (Hau and Skovgaard Jensen, 1987).

At E12.5, pregnant mice were identified by determining their weight gain, with at least 20% weight gain considered a lower limit for inclusion in the sub-

sequent treatment. Mice determined to not be pregnant were group housed for several days, then subjected to the timed mating procedure again.

E12.5 was selected for the administration of Poly(I:C) since the effect of Poly(I:C)-induced MIA on depression-related behaviours and associated neurobiological changes in the mouse has been previously described at this time point (Khan et al., 2014).

Pregnant mice were injected intraperitoneally (i.p.) with 20mg/kg Poly(I:C) (Polyinosinic:polycytidylic acid potassium salt, Sigma-Aldrich) dissolved in vehicle or the vehicle alone, physiological saline solution (0.9%, Fresenius Kabi). The injection volume for both conditions was 10 ml/kg.

Following the administration of Poly(I:C) or vehicle, the dams were placed back in their home cage and given a paper towel to create a nest. At birth, pups were counted, then left with the mother until they reached the age of 3-4 weeks.

2.1.4   Weaning of offspring

The offspring were weaned from their mother at the age of 3-4 weeks: The sex of each mouse was determined and the litter-mates were subsequently housed in group cages of up to 5 mice segregated by sex.

2.1.5   Brain tissue extraction

At age 8-9 weeks, a period corresponding to young adulthood in humans, all mice were sacrificed by cervical dislocation. The brains were extracted quickly and the hippocampi were dissected bilaterally from each animal.

Western Blot experiments, mRNA analysis and chromatin immunoprecipitation (ChIP) were carried out using tissue from parallel cohorts of mice.

For ChIP and Western Blot experiments, the tissue was placed in reaction

tubes and flash-frozen using liquid nitrogen immediately following dissection, and then stored at $-80°$C until used for analysis. mRNA samples were stored in RNAse-free reaction tubes containing RNAlater (Ambion) and stored at $-20°$C for subsequent experimental work-up.

2.2   Molecular biology experiments

2.2.1   Western Blot analysis of hippocampal SERT levels and hippocampal H3 and H4 acetylation levels

Standard Western Blot Analysis was performed on hippocampal tissue of Poly(I:C)-treated and control mice after first homogenising the tissue in 1M PBS, then lysating this in a protein lysis buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% SDS, 0.5% Triton X-100, 1 mM EDTA, 10 mM NaF, 5 mM $Na_4O_2P_7$, 10 mM Na_3VO_4 and 1x protease inhibitor cocktail (Thermo-Scientific)) for the assessment of SERT and histone H3 and H4 (total and acetylated) protein levels.

Protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology) according to the manufacturer's instructions and using a Synergy Multi-Mode Microplate Reader (Biotek) for spectroscopic measurement. Western Blot experiments followed a standard protocol (Griesauer et al., 2014). Briefly, for each sample, 50 μg of total protein was mixed with 5μl Loading Buffer (ThermoScientific) and loaded onto a 10% polyacrylamide gel and subjected to SDS-PAGE electrophoresis (70V, 2:30 hours). Subsequently, proteins were transferred to a membrane (Immobilon-P Membrane, Millipore), blocked for 30 minutes at room temperature (RT) under constant shaking using TBST (Tris-Buffered Saline and Tween 20) buffer containing 5% dried milk (Sigma-Aldrich). After washing the membranes with TBST

three times, they were incubated with the appropriate primary antibodies (SERT: 1:1000, Santa Cruz; β-actin: 1:2000, US Biological; H3: 1:1000, Abcam; acH3: 1:1000, Millipore; H4: 1:1000, Millipore; acH4: 1:1000, Millipore) overnight at 4°C. The next day, membranes were washed 4 times with TBST (5 minutes each), followed by a one-hour incubation on a shaker at RT with the appropriate secondary antibody (SERT: Donkey anti-goat IgG-HRP, 1:3000, Santa Cruz; β-actin: Goat anti-mouse IgG-HRP, 1:3000, Cell Signaling Technology; all histones: anti-rabbit IgG-HRP, 1:3000, Cell Signaling Technology) and another three 5-minute washes with TBST. Pierce ECL Western Blot Substrate (Pierce Biotechnology) was used to develop the membranes according to manufacturer's instructions. Quantification was performed by chemiluminescent imaging with a FluorChem HD2 (Alpha Innotech) using the respective software. Values obtained from densitometry of target proteins (SERT, acetylated H3 and H4) were normalised (β-actin for SERT, total H3 and H4 for acetylated H3 and H4) for the semiquantitative determination of protein levels.

2.2.2   mRNA expression analysis of SERT and HDACs: RNA isolation, cDNA synthesis and qRT-PCR

mRNA was extracted using the RNEasy Mini Kit (Qiagen) according to the protocol supplied by the manufacturer.

Subsequently, cDNA synthesis was carried out using the DyNAmo cDNA Synthesis Kit (ThermoScientific) following the manufacturer's instructions. Briefly, 900 ng of mRNA were transferred to an RNAse-free PCR reaction tube and RNase-free water was added to reach a total volume of 7 μl. Following this, 13 μl of master mix (10 μl reverse transcriptase buffer, 1 μl random hexamer primer set, and 2 μl M-MuLV RNase H+ reverse transcrip-

tase) were added to each sample, resulting in a total reaction volume of 20 μl. In addition, minus reverse transcriptase controls were prepared for each sample, using all components but the M-MuLV RNase H+ reverse transcriptase, to control for contamination of the mRNA sample by genomic DNA. cDNA samples were amplified using the following program settings: 10 minutes of primer extension at 25°C, 30 minutes of cDNA synthesis at 37°C, termination of the reaction for 5 minutes at 85°C, and finally the samples were cooled to 4°C.

Finally, qRT-PCR was performed with the cDNA samples (dilution 1:5) to determine the relative levels of hippocampal mRNA (SERT, HDAC 1-11). For each sample, 7.5 μl of SYBR Green MasterMix (LifeTechnologies), 0.5 μl each of forward (SERT: TATCCAATGGGTACTCCGCAG, Invitrogen) and reverse (SERT: CCGTTCCCCTTGGTGAATCT, Invitrogen) primers, 2.2 μl of RNase-free water and 5 μl of sample were added to a well of a RT-PCR plate. All reactions were carried out using technical duplicates.

For each sample, C(t) values were obtained for the target gene as well as for β-actin in a parallel control reaction (β-actin forward: ATGGTGGGAATGGGTCA-GAAG; reverse: TCTCCATGTCGTCCCAGTTG, Invitrogen).

The C(t) values for β-actin were used for calculation of Δ C(t) - representing the relative quantification of target mRNA amounts in each sample, further allowing the calculation of $\Delta\Delta$ C(t) which expresses the fold change of mRNA levels observed between Poly(I:C)-exposed mice and untreated mice, using the formula:

$$2^{-(\Delta\Delta C(t))}$$

To obtain the standard errors of the mean (SEMs) of these values, SEMs were first calculated from $\Delta\Delta$ C(t). Next, the lower and higher limits of the mean $\Delta\Delta$ C(t) values $-/+$SEM were calculated, and with each of these the

corresponding lower and higher limits of the mean fold changes $(-/+\text{SEM})$ using the formula $2^{-(\Delta\Delta C(t))}$. The differences between these values and the mean $2^{-(\Delta\Delta C(t))}$ for each group were used as the SEMs in the graphical representations.
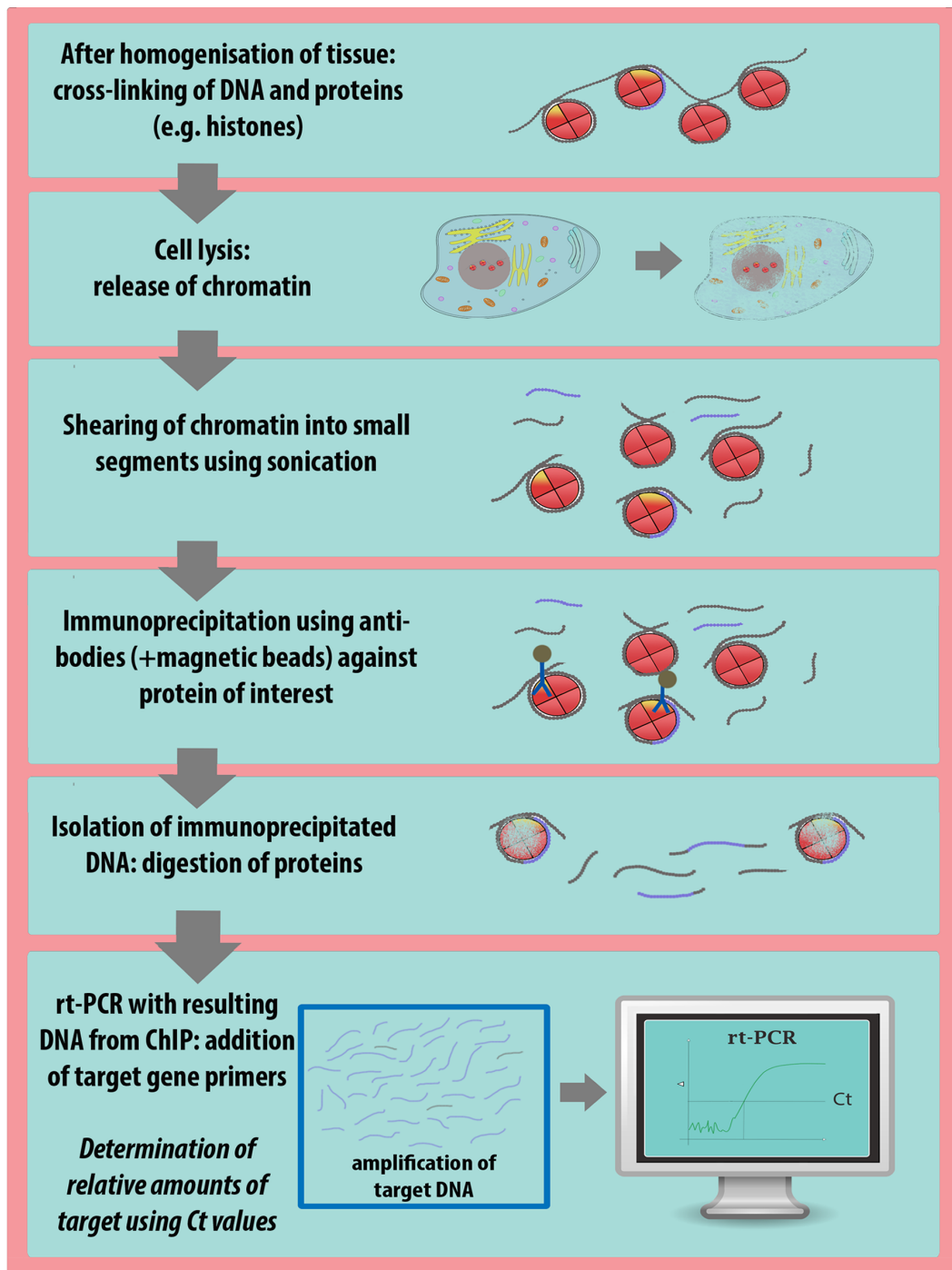
2.2.3   Chromatin immunoprecipitation for determination of H3 and H4 acetylation levels at the SERT locus in the hippocampus

Chromatin immunoprecipitation (ChIP) was performed to determine levels of H3 and H4 acetylation at the SERT promoter in mice subjected to maternal immune activation and control mice. Because of the relatively large amount of tissue needed for ChIP, hippocampal samples originating from two mice of the same treatment group and sex were pooled to obtain one ChIP sample. The pooling of samples was performed randomly across different litters to eliminate any possible litter effects, which have been shown to occur due to differences in maternal behaviour and postnatal environments (Curley et al., 2009).

The following protocol for "fast ChIP", adapted from Nelson et al. (2006), was optimised during the course of this research project. An outline of the crucial steps in this method is available in Figure 3.

Hippocampal tissue was homogenised in 1.42% formaldehyde in 500 μl PBS (1M) using a hand homogeniser, after which it was left to cross-link for 15 minutes at room temperature. The cross-linking reaction was quenched by adding glycine (125 mM) and leaving the reaction for 5 minutes at room temperature. Following this, the samples were washed three times with 1 ml ice cold PBS, centrifuging the samples for 5 minutes (2000G, 4°C) between the washes. After the removal of PBS, 1 ml of low salt IP buffer with pro-

Figure 3. Important steps in the chromatin immunoprecipitation (ChIP) assay. The essential steps of ChIP and their function are described.

tease inhibitors (IP Buffer, low salt: 150 mM NaCl, 50 mM Tris-HCl / pH 7.5, 5 mM EDTA, 0.5% NP-40, 1% Triton X-100; Halt protease inhibitors added just prior to use in 1:100 dilution, ThermoScientific) was added to each sample for cell lysis, the sample was resuspended, vortexed, and left for 5 minutes on ice. After cell lysis, the samples were centrifuged again for 5 minutes (2000 G, 4°C) and washed once with low salt IP buffer with inhibitors. After removing this by centrifuging once again (5 min, 2000G, 4°C), the sample was finally resuspended in 500 μl low salt IP buffer with inhibitors. The chromatin of all samples was then sheared using a sonicator bath (Bioruptor Plus, Diagenode; Settings: high intensity, 30 cycles: 30 seconds on, 30 seconds off) to obtain small ($\leq$1000 base pairs) fragments of chromatin. The samples were then divided into three equal parts of 150 μl: Two for immunoprecipitation with antibodies (anti-acetyl-H3, undiluted, Millipore; and anti-acetyl-H4, undiluted, Millipore) and one for mock immunoprecipitation (without antibodies). Each IP sample volume was increased to 800 μl (using low salt IP buffer with protease inhibitors) before adding 7 μl of antibody to acH3 and acH4 samples and 20 μl of protein A magnetic beads (Magna ChIP Protein A Magnetic Beads, Millipore) to all samples. All IP samples were incubated overnight at 4°C on a rotating platform, ensuring that the magnetic beads stayed in suspension throughout this incubation period.

The following day, the samples were washed with ice cold buffers a total of six times: twice with 1 ml low salt IP buffer, twice with high salt IP buffer (as low salt except 500 mM NaCl), and again twice with low salt IP buffer. After each wash, a magnetic rack (Magna GrIP Rack, Millipore) was utilised to pellet the samples for easy removal of the buffer. After removal of the buffer after the final wash, the DNA was extracted as follows: The pellet was resuspended in 100 μl PBS (1M) and 1 μl of proteinase K (20 ug/ul)

was added to digest the proteins bound to the DNA. The samples were then placed for 30 minutes on a heated shaker (55°C) to activate the proteinase K, and at high speed (1200 rpm) to ensure the continued suspension of the magnetic beads. Then the samples were boiled at 95°C for 15 minutes to in-activate the proteinase K. The now PCR-ready DNA samples were isolated by using the magnetic rack once again to remove the magnetic beads, and transferred to new reaction tubes. These samples were stored at - 20°C until use in qRT-PCR.

For qRT-PCR, master mixes containing SYBR Green Master Mix (12.5 μl per sample repeat, LifeTechnologies) and SERT primers (4 μl each, sequences forward: CAGAGCTCTCAGTCTTGTCTCC; reverse: TGCTG-GTCAGTCAGTGGTG; Invitrogen) were prepared. 4.5 μl of each sample was added per reaction. Each reaction was repeated twice within the same PCR plate.

C(t) values for each sample and IP condition were recorded. The analysis of these ChIP experiments involved the calculation of Δ C(t) similar to above, allowing the calculation of the relative abundance of material contained in the IP samples in relation to the mock IP sample, which accounts for tissue amounts and non-specific binding of the magnetic beads in each IP. Further, $\Delta\Delta$ C(t) was calculated to compare levels of detected proteins at the SERT promoter between MIA-exposed and control groups, allowing the calculation of fold changes relative to the control levels of the protein using the formula:

$$2^{-(\Delta\Delta C(t))}$$

To obtain the SEMs of these values, SEMs were first calculated from $\Delta\Delta$ C(t). Next, the lower and higher limits of the mean $\Delta\Delta$ C(t) values $-/+$SEM were calculated, and with each of these the corresponding lower and higher limits of the mean fold changes $(-/+$SEM) using the formula $2^{-(\Delta\Delta C(t))}$. The

differences between these values and the mean $2^{-(\Delta\Delta C(t))}$ for each group were used as the SEMs in the graphical representations.

2.3   Statistical analysis

Two-sample t-tests (two-tailed, equal variance) were carried out in Microsoft Excel to test for significant differences between groups for each of the results of the experiments described above. P values were considered statistically significant when p $\leq$0.05. In particular, for the results of qRT-PCR experiments (SERT and HDAC mRNA, ChIP), the calculated $\Delta\Delta$ C(t) values were used in the t-tests, since the fold change values $(2^{-(\Delta\Delta C(t))})$ used in the graphical representations of the results do not follow a normal distribution.

41

3. RESULTS

3.1 MIA alters hippocampal SERT expression

3.1.1 MIA reduces hippocampal SERT protein levels

In light of the important link between SERT and the aetiology of depression (Haase and Brown, 2014), we evaluated protein and mRNA expression levels of SERT in the hippocampus of the adult MIA-challenged mice.

Western Blot analysis revealed significantly lower SERT protein levels in the hippocampus of adult mice subjected to MIA during development (n=5) compared to that of control mice (n=5; p$\leq$0.01; Figure 4 a and b).

3.1.2 MIA reduces hippocampal SERT mRNA levels

To investigate whether the observed change in SERT protein levels results from alterations in the transcriptional machinery of this gene, levels of SERT mRNA were next examined. qRT-PCR analysis showed an approximately 50% downregulation of SERT mRNA levels in hippocampal tissue of adult MIA-exposed individuals (n=10) as compared to control animals (n=9) (p$\leq$0.0001, Figure 5).
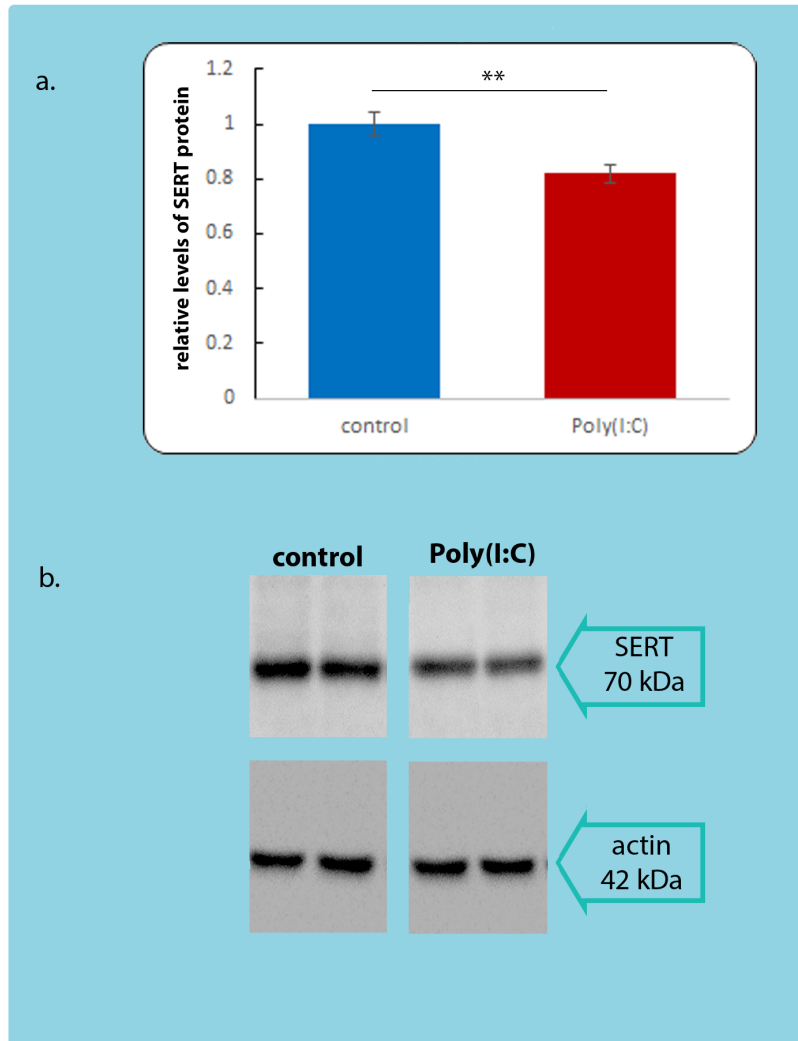
Figure 4. SERT protein levels in the hippocampus after MIA. Graph (a) represents relative levels of SERT expression in the hippocampus of adult control vs. Poly(I:C)-exposed mice (n=5 per group), normalised to control protein levels, and (b) shows representative images of the Western Blot. [data displayed as mean +/- SEM, ** denotes $p \leq 0.01$.]
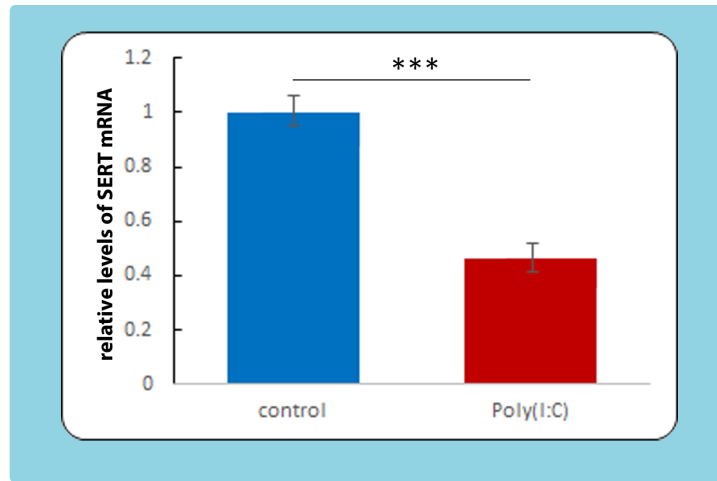
Figure 5. Relative hippocampal expression levels of SERT mRNA after MIA. The graph shows the levels of SERT mRNA in the adult offspring of control animals (n=9) vs Poly(I:C)-injected individuals (n=10), normalised to average control mRNA levels. [data displayed as mean +/- SEM, *** denotes p ≤ 0.001]

3.2   MIA alters the global epigenetic profile in the hippocampus

3.2.1   MIA reduces total hippocampal H3 and H4 acetylation levels

To determine the relative abundance of total and acetylated histones H3 and H4 hippocampus of MIA-exposed individuals and control mice, Western Blot analysis was carried out in order to investigate whether MIA induces epigenetic regulatory mechanisms in a brain region implicated in depression.

Semiquantitative analysis of bands (n=3 for each group) revealed no significant difference in the ratio of acetylated H3 vs. total H3 in the hippocampi of the adult MIA-treated vs. untreated offspring (Figure 6 a and b).

For acetylated H4 relative to total H4 a significant decrease in relative levels of acetylated H4 in adult MIA-exposed individuals was observed (p≤0.05, Figure 6 c and d), suggesting a global reduction in H4 acetylation in the
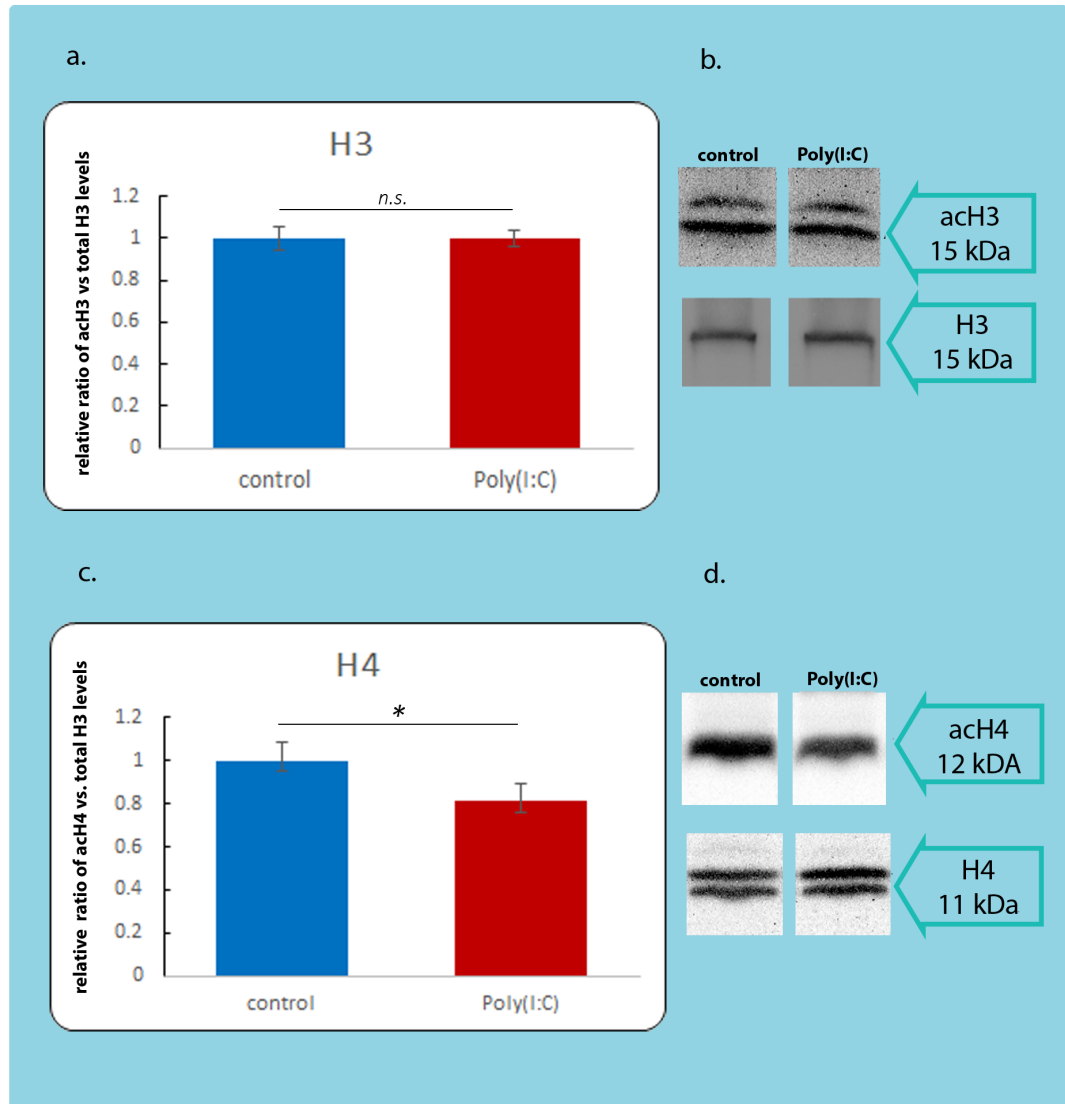
Figure 6. Comparison of relative levels of acetylated H3 and H4 vs. total H3 and H4 in the hippocampus of adult control and MIA-exposed offspring. Graphs show the quantification of protein levels for the MIA-exposed offspring (n=3) relative to control (n=3) group (a, c) and representative images of the Western Blots (b, d) are provided. [data displayed as mean +/- SEM, n.s. denotes p>0.05, * denotes p ≤ 0.05]

hippocampus by exposure to Poly(I:C) during embryonic development.

3.2.2   MIA influences the hippocampal levels of two HDACs

To further delineate how MIA may act to induce epigenetic regulation in the hippocampus of adult offspring, qRT-PCR examining the expression levels of histone deacetylating enzymes (HDACs) (1-11), which influence overall histone acetylation levels as well as gene-specific histone acetylation (Lombardi et al., 2011), was undertaken. Figure 5 (a-g) shows a graphical representations of the relative levels of each detectable HDAC mRNA in the adult offspring hippocampus of control (n=7) vs. MIA mice (n=8). Hippocampal mRNA levels of HDAC 2 and HDAC 9 were significantly decreased following exposure to a maternal inflammatory state (p≤0.05, Figure 7 b and g) while no alterations in transcript levels of the remaining HDACs were observed (Figure 7 a, c, d, e and f). Of note, levels of HDAC 6, 8, 10 and 11 were at the limit of detection in our samples, thus hampering proper statistical evaluation.

3.3   MIA alters the level of acetylated H3 and H4 at the SERT promoter

To investigate whether global alterations of hippocampal histone acetylation were also paralleled by respective specific events at the SERT gene which could account for the observed changes in SERT expression in adult offspring after MIA exposure, acetylation levels of H3 and H4 were examined locally at the SERT promoter.

Chromatin Immunoprecipitation (ChIP) analysis showed that hippocampal levels of acetylated H3 at the SERT promoter were reduced by about 50% in
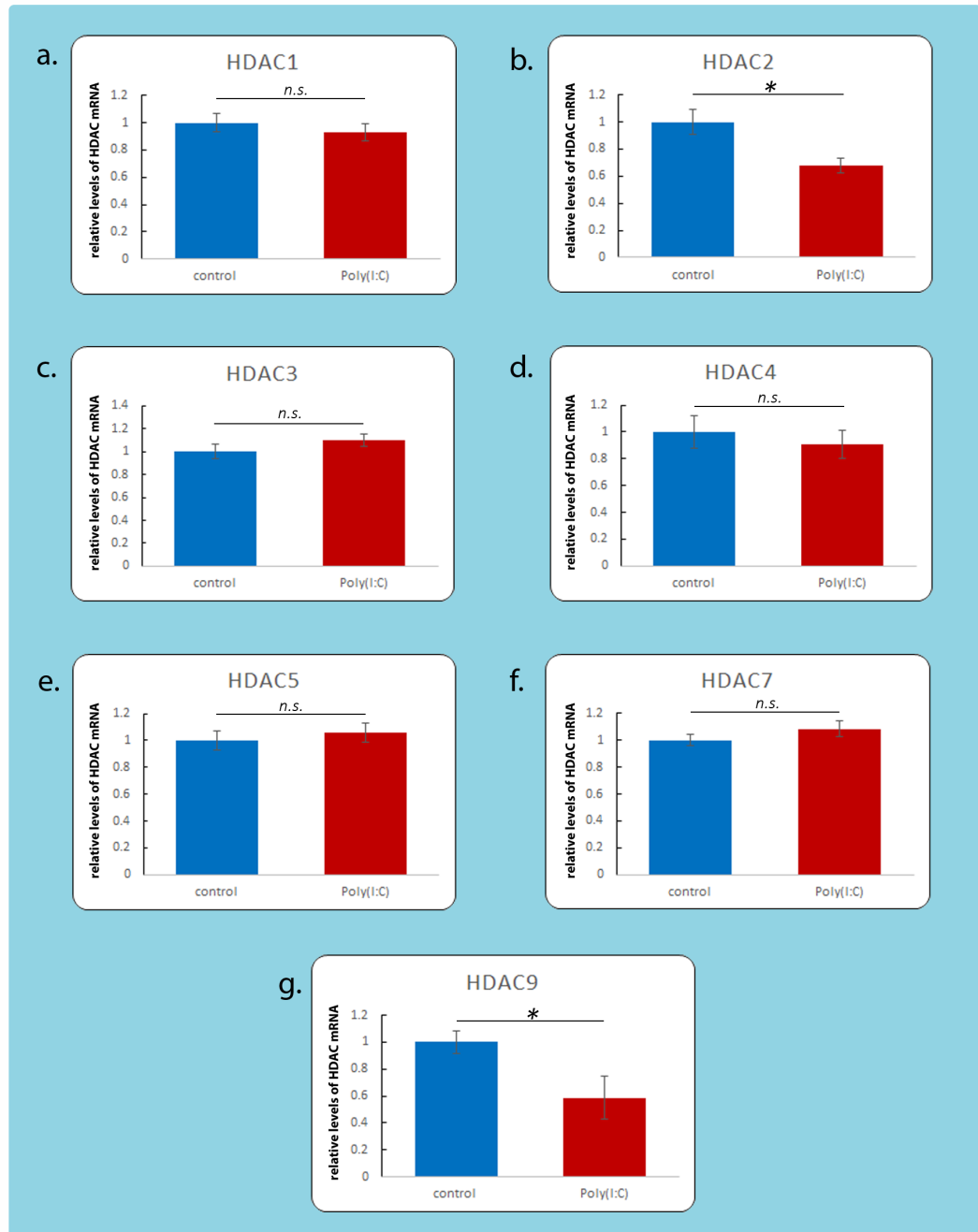
Figure 7. Relative hippocampal levels of different HDAC mRNA in adult control and MIA-exposed mice. HDAC mRNA levels, relative to control levels, are shown for each of HDAC 1 (a), 2 (b), 3 (c), 4 (d), 5 (e), 7 (f) and 9 (g). Sample sizes were n=7 for the control group and n=8 for the Poly(I:C)-exposed group. [data displayed as mean +/- SEM, * denotes p ≤ 0.05, n.s. denotes p > 0.05].
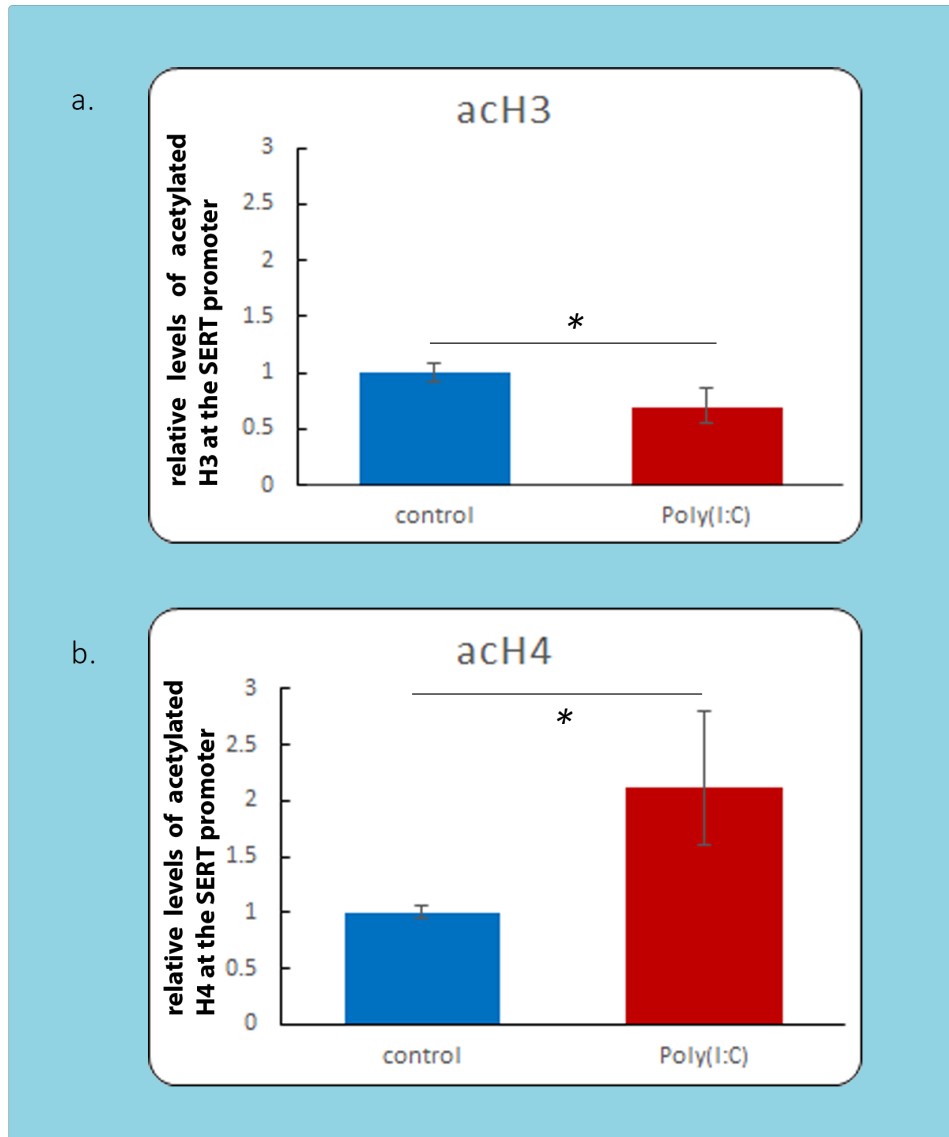
Figure 8. ChIP: Relative levels of acetylated H3 (a) and H4 (b) histones at the SERT promoter in the hippocampus of control vs. MIA-exposed mice. Graphs show the relative amounts of acetylated H3 and H4 histones observed in association with the SERT promoter in hippocampal samples of the offspring of vehicle- and Poly(I:C)-treated mice (n=12 per group). Relative amounts are normalised to mock IP values and the control group mean. [data displayed as mean +/- SEM, * denotes p ≤0.05]

49

Poly(I:C)-exposed offspring compared to control offspring (n=12 per group) (Figure 8 a). Furthermore a significant, two-fold increase of acetylated H4 at the SERT promoter was observed in Poly(I:C) offspring compared to the control group (p≤0.05, Figure 8 b).

4. DISCUSSION

In an attempt to elucidate the molecular nature of the long-term impact of gestational infection on brain structure and function at the molecular, cellular and behavioural level related to depression, reproduced in the Poly(I:C)-induced mouse model of MIA, we here examined the potential involvement of epigenetic regulatory mechanisms, focusing specifically on SERT.

4.1   The effect of MIA on SERT levels in the adult offspring hippocampus

We first decided to investigate the effect of MIA on SERT expression in adult offspring, considering the proposed pivotal role of SERT in the pathological mechanisms leading to the development of MDD (Haase and Brown, 2014) and its central importance as a drug target in antidepressant pharmacotherapy. We therefore first examined SERT expression in adult MIA-challenged mice, focusing on the hippocampus, a brain region central to the neural circuitry of depression (Small et al., 2011) and found a significant reduction both at the protein and mRNA levels in MIA as compared to control offspring.

At first sight, this result may seem counterintuitive when considering the "classical" monoaminergic theory of depression, which is based upon the

assumption that a dysbalance in serotonergic neurotransmission plays an integral role in the pathophysiology of depression and that long-term treatment with pharmacological antidepressants, mainly SSRIs, serve to restore deficient serotonergic function. Accordingly, it had been proposed that a reduction of SERT activity, as resulting from administration of SSRIs, would increase the availability of serotonin at the synapse and initiate an antidepressant response (Schildkraut, 1965). However, since the therapeutic effect of SSRIs only emerges after chronic treatment (Gelenberg and Chesen, 2000), it is still relatively unclear how the acute effect of SSRIs on the serotonergic synapse relates to their antidepressant activity after prolonged treatment.

Together these observations illustrate that the relationship between SERT levels and depression-related phenotypes or symptoms is not as simple as acute SSRI treatment and analogous experiments may suggest, and this is additionally supported by the dichotomy between effects of acute SERT inhibition and those of a developmental lack or reduction of SERT, as can be seen in knock-out (KO) models of SERT in transgenic rodents: Several studies, described below, have investigated the effect of developmental manipulation of SERT, and have mostly found effects that appear to oppose the general idea that a reduction in SERT function equates with an antidepressant effect. Firstly, SERT KO mice show marked increases in several depression-related behaviours, while not showing any obvious neurodevelopmental defects (Lira et al., 2003; Gardier et al., 2009). This result indicates that SERT (and dependent serotonergic signalling mechanisms) may have an important role during brain development that is relevant to the proper maturation of neuronal circuits regulating mood, while also being important for the regulation of acute serotonin availability at the synapse.

Both SERT KO mice and mice heterozygous for a SERT deletion show sig-

nificantly increased baseline serotonin levels in the extracellular space along with their behavioural alterations (Shen et al., 2004). Considering that this is also the situation after acute SSRI administration, this corroborates the ambiguous role for serotonin and SERT in the pathological mechanisms of MDD, depending on the timing of SERT disruption. It also highlights the fact that developmental manipulations may result in some form of adaptory mechanisms that may compensate for the loss of specific gene functions, possibly explaining why seemingly contradictory effects are observed in developmentally induced vs. adult rodent models related to MDD (Kalueff and LaPorte, 2010).

For example, maternal separation in the early postnatal period, a model of early life stress that is known to induce depression-related behaviour, leads to a reduction in SERT mRNA levels in the dorsal raphe nuclei in adulthood (Bravo et al., 2014). Considering that hippocampal serotonergic neurons originate in the raphe nuclei of the brainstem, it is likely that these reduced levels would be also reflected in the hippocampus, which yet has to be investigated, but would suitably confirm our own results in the MIA model. Thus two detrimental early life events of quite different natures, namely infectious versus psychosocial stress, appear to have a parallel effect on SERT expression later in life, and are both accompanied by depression-related behaviours. Along these lines, pharmacological blockade of SERT during early life using SSRIs led to depression-related behaviours in adult mice comparable to those of constitutive SERT KO mice (Vogel et al., 1990; Ansorge et al., 2004). This suggests that the effect of SERT KO on mood-related phenotypes stems from the role of SERT in brain development, likely in the maturation of neuronal circuits related to emotional processing, independently of the later role of SERT in the organism (Ansorge et al., 2004), which also explains the differ-

ence in the effects of SERT blockade in adulthood.

Taken together, animal models of MDD that involve developmental manipulations are associated with a long-term reduction or absence of SERT expression, supporting the findings of the present study. Interestingly, these results obtained in animal models are also supported by studies examining SERT levels in depressed individuals which observed a significant reduction of SERT in specific brain regions, as reported in a recent meta-analysis of human imaging studies (Gryglewski et al., 2014). However, it is also pointed out that no study had enough power by itself to detect the relatively small effect observed, indicating that more experiments are needed for independent validation of these results (Gryglewski et al., 2014).

To recapitulate, the presented results together with evidence from the literature collectively suggest that in both developmental environmental and genetic animal models, reduced and/ or absent SERT expression is associated with depression- and anxiety-related phenotypes, which is in opposition to other avenues of research into MDD that are based upon the known acute effect of SSRIs on SERT function and mood. This seemingly dual role of SERT in relation to depression-like behaviour may be due to the important role that serotonin plays in the development of the CNS where serotonin acts as a trophic factor, instructing synaptogenesis, axonal outgrowth and dendritic pruning, all of which are essential for the correct wiring of the brain (Andersen and Navalta, 2004). An alternative explanation may be that the organism manages to adapt to and/or compensate for a lack of SERT and the accompanying increase in synaptic serotonin during development. Further experiments are needed to clarify the alterations that may take place to compensate for dysfunctional serotonergic neurotransmission related to alterations in SERT expression during development. For example, a study of

54

SERT levels at several developmental stages after MIA by Poly(I:C) would demonstrate whether the reduction in SERT that we observed is already present during development, or alternatively, whether time-dependent variations of SERT levels occur during the course of brain development. Concurrently, one could examine the effect on SERT function by carrying out efflux studies, to examine if the changes in SERT expression are accompanied by functional differences in serotonin transport.

4.2   MIA-induced global epigenetic regulations in the adult offspring brain

Trying to understand how MIA can "program" life-long alterations in behaviour and protein expression, we set out to examine its effect on elements of the molecular machinery involved in epigenetic regulation. To this end, we focused on relevant general epigenetic markers, namely the relative acetylation levels of H3 and H4 and the expression of HDACs in the hippocampus of adult MIA-exposed offspring.

We first examined posttranslational modifications of histones which are associated with transcriptionally active and/or repressed states by determining acetylation of histone H3 and H4. Both histones are highly implicated in the pathophysiology of depression and have been repeatedly proposed as molecular effectors mediating long-lasting *Gene × Environment* interactions (Liu et al., 2008; Tammen et al., 2013). We here observed a significant decrease in H4 acetylation in hippocampal tissue of MIA offspring which would correspond to an increase in the binding of H4 to DNA, hence precluding the accessibility of RNA polymerases to relevant promoter regions and thereby

decreasing gene transcription (Shahbazian and Grunstein, 2007). This finding is specifically novel since most studies investigating histone acetylation in association with a depression-related phenotype have used stress paradigms applied in the early postnatal period or in adulthood, and have most often found a decrease in H3 acetylation, while the only study investigating the effect of postnatal stress on H4 acetylation found a significant increase in stressed rodents (reviewed in Bagot et al. (2014)).

Histone acetylation resulting from MIA has so far only been investigated in one other study, in which a global hypoacetylation of H3 and H4 in cortical tissue but no significant differences in H3 or H4 acetylation in the hippocampus were reported in the brains of juvenile mice exposed to prenatal Poly(I:C) (Tang et al., 2013). These effects were not observed in adult mice, though a trend for reduced H4 acetylation in adult mice was noted (Tang et al., 2013). These findings lend support to our results and together propose that MIA induces a reduction in H4 acetylation in the hippocampus of adult offspring possibly associated with an inhibitory constraint on gene expression which may be involved in the emergence of the depression-like behavioural phenotype.

The observed global decrease in hippocampal histone acetylation is most likely a result of differential levels and/ or function of the enzymes responsible for catalysing this reaction, histone acetyl transferases (HATs) and histone deacetylases (HDACs), commonly thought to regulate histone acetylation levels (Peserico and Simone, 2011). Here we investigated the expression of members of the HDAC superfamily, previously shown to be of relevance in the pathophysiology of depression (Schroeder et al., 2007; Hobara et al., 2010), by setting out to determine mRNA levels of HDAC 1-11 in hippocampal tissue of adult MIA and control offspring. We observed a specific and significant

reduction of HDAC2 and HDAC9 expression in MIA-challenged mice, while no changes in mRNA levels of the other HDACs was found. However, it has to be noted that results for four HDACs (6, 8, 10, 11) were inconclusive due to the limited expression of these transcripts in our samples.

While this is the first report on the expression of HDACs in brains of adult offspring after prenatal immune activation, there are numerous lines of evidence pointing towards an important involvement of HDACs in the molecular pathomechansims associated with depressive-like mood changes both in human patients and pertinent animal models. For instance, during a depressive episode MDD patients showed increased plasma levels of HDAC2 and HDAC5 compared to healthy controls, which normalised when patients went into remission, suggesting that aberrant expression of these HDACs is related to acute depressive episodes (Hobara et al., 2010). In the social defeat stress model, a chronic paradigm that induces depression-like behaviour in adult mice, hippocampal infusion of a class I HDAC inhibitor reversed the observed increase in anhedonia-like behaviour in defeated mice, suggesting that deacetylation by one or several HDACs of class I (which encompasses HDACs 1, 2, 3 and 8) is involved in the development of the depression-like behaviour in this paradigm (Covington et al., 2011).

Paralleling this observation, it was shown that chronic imipramine (TCA) administration after social defeat stress led to a reduction in HDAC 5 levels in the hippocampus, and that this effect was fundamental for the success of the treatment, whereas vector-mediated overexpression of HDAC5 blocked its effects - suggesting that HDAC5-mediated deacetylation counteracts the antidepressant effect of imipramine (Tsankova et al., 2006). Indeed, it was recently reported that various antidepressant and mood-stabilising drugs have significant effects on the levels of HDACs (including HDAC2), in several re-

gions of the brain, supporting the notion that HDACs may be relevant for the action of mood-related pharmacological treatments (Ookubo et al., 2013) and/ or themselves mediate neurobiological mechanisms conveying the corresponding behavioural effects as proposed by another recent study (Schroeder et al., 2013). Collectively, these findings support the idea that increased histone acetylation and therefore modification of the acetylation content of nucleosomes and its regulation by HDACs is important in molecular mechanisms bi-directionally modulating mood-related behaviours. However, the here observed decrease in hippocampal HDAC 2 and 9 transcript levels in adult MIA offspring appears to contrast the presented previous reports in the literature, further supporting the notion that the relationship between histone deacetylation and depression-like behaviour may be more complex and may critically depend on the specific genetic and/or environmental condition inducing the particular phenotype. One obvious and relevant difference between ours and other paradigms, as mentioned above, is the timing of the environmental insult - MIA impacts on brain development during gestation whereas *adult* mice are subjected to the social defeat paradigm, which dominates MDD-related research on HDACs - and this may contribute to the observed differences.

Indeed, an early stress paradigm, which subjects rodents to environmental stress in the postnatal period, led to a significant downregulation of several HDACs - including HDACs 2 and 9 - in the adult hippocampus (Suri et al., 2014), supporting our own results. This could potentially suggest that the dysregulation of these particular HDACs in this brain region is specifically linked to environmental insults endured during development.

Hence it can be speculated that interference with embryonic brain development would induce compensations and adaptations which are not observed

in non-developmental models of MDD, but which may be particularly aetiologically relevant. While a reduction in HDAC 2 and 9 expression may not account for the observed decrease in global hippocampal H4 acetylation and histone-mediated epigenetic regulations in MIA offspring, it could relate to the pathomechanisms of MIA-induced depression-like behaviour through different pathways. For example, HDAC2 was shown to be upregulated in neural progenitors in the adult mouse hippocampus specifically as the cells differentiate (MacDonald and Roskams, 2008). Hence, this finding indicates that the previously reported reduction in adult neurogenesis in the hippocampus of MIA offspring (Khan et al., 2014) could potentially also be related to the reduced levels of HDAC2 we observed herein.

As for HDAC9, which belongs to the type IIa class of HDACs, members of which appear to exhibit little or no histone deacetylation activity, but are thought to exert their transcription-regulatory effect by forming complexes with other proteins (Parra, 2014), this enzyme has not been previously associated with depression or related animal phenotypes. Interestingly, however, accumulating evidence points towards and important role for HDAC9 in neuronal development and severe mental illnesses, as more reports on the potential involvement of this HDAC in the pathomechanisms of schizophrenia and ASD, both of which are critically linked to gestational infection, are emerging (Parra, 2014; Pinto et al., 2014; Lang et al., 2012). Thus, considering our findings on aberrant expression of HDAC9 in light of the popular use of the Poly(I:C) paradigm as MIA model for schizophrenia and autism (Meyer et al., 2009), future research investigating the specific relevance of this HDAC for the consequences of infections during pregnancy on brain structure and function may lead to important insights into the pathophysiology of associated psychopathologies, as well as depression.

59

With regards to the observed global decrease in hippocampal H4 acetylation in MIA offspring, it is unlikely that the alterations in HDAC2 account for the underlying molecular mechanisms, suggesting the involvement of alternate regulatory principles. Promising candidates in this regard would be HATs, which modulate epigenetic changes in gene transcription through histone acetylation. However, much less is known about their potential involvement in the brain and behavioural alterations related to neuropsychiatric disorders than in the case of HDACs, and even less in relation to depression.

Collectively our data on central players involved in histone-dependent epigenetic regulations lend support to the notion that MIA impacts important epigenetic processes in the adult hippocampus offspring which might contribute to the observed long-lasting molecular, cellular and behavioural consequences of gestational infections.

4.3 Specific epigenetic regulation at the SERT promoter in the hippocampus of MIA offspring

In addition to examining global epigenetic regulations in the hippocampus of MIA offspring, we also investigated gene-specific events, focusing on SERT, since reduced hippocampal SERT mRNA and protein levels were determined in MIA offspring. Hence, in an attempt to further clarify the nature of the epigenetic alterations influencing SERT expression caused by MIA, we next aimed to examine hippocampal H3 and H4 acetylation levels at the SERT promoter in MIA and control offspring.

Using ChIP analysis we observed a significant and specific decrease of relative H3 acetylation, as well as an increase in H4 acetylation at the SERT promoter in the hippocampus of individuals that underwent MIA during de-

velopment.

In general, as mentioned above, it is widely accepted that increased histone acetylation favours the transcription of genes through several mechanisms, one of which is the promotion of a more open structure of the chromatin (Turner, 2014). However, although regarded by some as unequivocal, this hypothesis is strongly contested by some and does not entirely reflect the findings of the last few decades of epigenetic research (Henikoff and Shilatifard, 2011; Swygert and Peterson, 2014; Shahbazian and Grunstein, 2007). Indeed, over 50 years ago experiments demonstrated *in vitro* that histone acetylation may reduce the inhibitory effect exerted on RNA polymerase due to a net decrease in positive charge carried by the modified lysine residue (Allfrey et al., 1964). This process is assumed to facilitate a looser chromatin structure by reducing the affinity of the acetylated histone residues to the DNA molecule, making the DNA more available to transcriptional initiation as well as digestion by DNase I (Lee and Lee, 2012; Turner, 2014). Today it is known that the mechanisms of transcriptional regulation by nucleosomes are far more complex than originally assumed, even if some aspects have been confirmed by modern methodologies (Shahbazian and Grunstein, 2007; Lee and Lee, 2012; Turner, 2014).

Considering the reduction in SERT transcript and protein levels and the parallel decrease and increase in acetylation of the different histones at the SERT promoter, our own results do not unequivocally support the hypothesis that increased histone acetylation at the regulatory region of a gene necessarily results in enhanced transcription thereof: Since we observed both increases and decreases in histone acetylation, the mechanism at hand cannot be as simple as one might assume. Though we found that H3 acetylation at the SERT promoter was reduced in MIA-exposed offspring, together with the

61

heightened acetylation of H4 that we observed, this cannot be interpreted to be the main mechanism underlying the observed decrease in SERT expression, and other explanations need to be sought. Similarly to our results, other studies have provided evidence for experimental observations in which transcript levels did not parallel levels of histone acetylation at the promoter of the gene in question.

For instance, when examining the effect of ECS (electroconvulsive seizures) on acetylation at several gene promoters, Tsankova et al. (2004) found that the cAMP response element-binding protein (CREB) promoter showed severalfold increases in H4 acetylation after acute ECS without a concomitant upregulation in CREB mRNA, suggesting that an alternative type of local regulation may be taking place which could potentially override the "activating" effect of hyperacetylation at this locus (Tsankova et al., 2004). Along these lines, one could speculate that the general H4 hyperacetylation at the SERT promoter in MIA offspring may be counteracted by other regulations occurring in parallel, including (but not limited to) the observed drop in H3 acetylation. Here, it is pertinent to consider further types of histones, namely H2A and H2B - which may also be acetylated at several residues (Bonenfant et al., 2006). Alternatively, a counteracting effect could originate from specific modifications of individual lysine residues of H4 at the SERT promoter which could be unacetylated or hypoacetylated in MIA offspring, thus contributing to a repression of gene expression, without however being detected in the ChIP procedure. If these residues happened to be at a particular position in H4, they may well have dominated over the effect of general H4 hyperacetylation at this locus: Indeed, post-translational modifications of histones, such as acetylation, have been shown to exert different effects depending on the position of the modified amino acid within the histone,

and several modifications - whether on the same histone molecule or not - can have different combinatorial effects on chromatin structure, recruitment of factors and consequentially transcription (Rothbart and Strahl, 2014). For example, genome-wide histone modification analysis has shown that a particular combinations of 17 histone modifications is associated with enhanced transcriptional activity (Wang et al., 2008), providing supporting evidence for a more complex view of transcriptional regulation. Thus a very detailed view of the particular modification pattern of H3 and H4 may be needed to investigate the link between histone acetylation and gene expression in depth. In this way, individual modifications may be sufficient to induce alterations in gene expression, while others may not contribute much to this type of regulation.

Furthermore, alternative approaches to the concept of a "histone code" have also been proposed: For example, some suggest that an attempt to interpret modifications to histones and DNA in an additive manner may be misguided, and instead propose that it is not, for example, acetylation itself that indicates gene activity but rather the dynamic and rapid cycling of acetylation and deacetylation is proposed as a mark of an active gene locus (Tsankova et al., 2007). While this type of cycling activity may prove more difficult to detect than a simple correspondence between acetylation levels and transcriptional activity, this possibility should be taken into account when attempting to interpret data concerning histone modifications in relation to gene expression.

Moreover, entirely different mechanisms, known to intervene in chromatin compaction and transcriptional regulation, such as DNA modifications, other histone modifications (methylation, phosphorylation, etc...) or the activity of chromatin-remodelling enzymes, could also be involved - creating ever more

63

possibilities for the subtle regulation of chromatin structure and function (Rothbart and Strahl, 2014; Swygert and Peterson, 2014).

The investigation of DNA methylation in particular is highly warranted, since previous research has shown that this kind of epigenetic modification at the SERT promoter may be relevant in MDD: For one, individuals with a lifetime history of MDD showed higher levels of methylation at the SERT gene, though this difference did not achieve statistical significance (Philibert et al., 2008). Further, methylation of SERT correlates with emotion-related brain function in the amygdala, suggesting it may be important in the regulation of mood-related aspects of SERT function (Nikolova et al., 2014), which we are primarily interested in. Another study showed that low methylation of SERT could predict the response of depressed patients to treatment with escitalopram, an SSRI, supporting the notion that knowledge of a patient's epigenetic status at the SERT gene may well be used in the future to personalise antidepressant treatment (Domschke et al., 2014). Considering the differential effects of SERT dysregulation in development and adulthood in addition to our results concerning SERT expression, as well as the putatively repressive role of DNA methylation, it would be interesting to look into the effect of MIA on DNA methylation at the SERT promoter.

Thus the concomitant investigation of other known epigenetic markers or mechanisms is needed to clarify the particular events in the case of MIA-induced alterations in SERT expression and associated epigenetic regulations at the SERT promoter.

Collectively, our findings further support the consideration of additional interpretations when attempting to correlate alterations in gene expressions with local epigenetic modifications. In particular, a focus on the dynamic cycling of histone modifications, as well as the role of individual histone and

DNA modifications that are necessary and sufficient to induce effects on gene transcription, represents a valid approach in this area of study.

5. CONCLUSIONS AND PERSPECTIVES

In conclusion, the present research project provides first evidence for alterations in several epigenetic processes in brains of the adult offspring resulting from MIA and proposes altered hippocampal SERT expression as one specific molecular effector.

The observed reduction of hippocampal SERT levels in adult MIA offspring postulates the hypothesis that SERT plays a critical role in the development of depression-related traits associated with gestational infections. These results further imply a differential role for SERT in depression-related phenotypes resulting from developmental impact and those arising as a consequence of endogenous and exogenous influencing factors experienced in adulthood. Collectively these observations strongly corroborate the pivotal role of the serotonergic system in the pathophysiology of depression, including an important role in the maturation of mood-related neural circuits.

The results of the experiments relating to epigenetic mechanisms offer a glimpse into the overwhelming complexity of epigenetic regulatory mechanisms, exemplarily demonstrated for histone acetylation and its relevance for the regulation of transcription, and shed some light on the potential role of these pathways in mediating the effects of MIA on adult offspring brain structure, function and related behavioural traits.

Giving one example, the present study - which represents, to the best of our knowledge, the first investigation of the effect of MIA on histone modifica-

tions at the SERT gene - found decreased H3 acetylation and increased H4 acetylation at SERT in the hippocampus of MIA offspring. This observation is however, considering the results in terms of SERT expression, in contradiction to the common doctrine postulating that hyperacetylation leads to upregulation of gene transcription and led us to re-examine this seemingly established assumption.

Hence, taking into account that the landscape of epigenetic regulations is by no means simple, linear and unidirectional, many questions remain unanswered, offering room to consider a variety of possibilities for interpretation of the results obtained, their inter-relationship and implications. This study, while offering some insight into the epigenetic systems modulated by MIA, has served as an even better tool to demonstrate that paradoxical findings must not be refuted, especially when dealing with a disease as multifactorial and complex as MDD.

With regard to this complexity, the validity of the Poly(I:C) model of MIA to study MDD needs to be acknowledged, while bearing in mind the advantages it brings to this area of study: Being a developmental animal model of depression, it is safe to assume that compensations - for instance in the affected neurotransmitter systems, including the serotonergic system - take place throughout the animal's brain development, and this is likely the reason that findings from developmental models appear to directly contradict the situation in adult models of depression. While this may at first seem like a confounding factor, it is important to remember that aetiologically, this may provide important information about the pathophysiological processes involved in MDD patients, since these compensatory process almost certainly also occurs in humans exposed to MIA. Thereby, the Poly(I:C) model of MIA presents the opportunity to study interactions between genes and the envi-

ronment, which are thought to characterise the aetiology of most complex diseases.

In the future, more detailed studies into the molecular mechanisms underlying the depressogenic effect of MIA are desirable, since this aspect of MIA and the Poly(I:C) model are often overlooked in favour of the (perhaps more obvious) association of MIA with schizophrenia - even though the burden on individuals and society by MDD far exceeds that of schizophrenia (Murray et al., 2012). A more detailed look at the epigenetic machinery at the SERT locus would allow a more valid interpretation of MIA-induced effects, while also adding to the general understanding of the nature of epigenetic regulation. Assays of serotonin uptake by SERT to elucidate whether the function of SERT is altered could provide further insight into the cellular effects of MIA.

Upstream of SERT, the mediators of the transcriptional regulation by MIA should be identified. One obvious candidate is IL-6, but it is unclear as of yet how this cytokine may regulate the expression of SERT. Since it is known that IL-6 activates the JAK-STAT pathway, inducing the translocation of the transcription factor STAT3 to the nucleus (Kishimoto, 2006), STAT3 may directly upregulate the expression of SERT. This hypothesis, constituting the underlying rationale for the PhD research project following the completion of this Master's thesis, would first have to be tested by inhibiting STAT3 under administration of IL-6, and examining SERT expression and concomitant depression-related behaviour, although the systemic effects of this inhibition may not allow any straightforward interpretation. To home in on the potential interaction between STAT3 and SERT, we therefore propose a mouse model generated using *Cre-Lox* recombinase technology, which

69

lacks the STAT3 gene in SERT-expressing neurons only: This region-specific knock-out of STAT3 would allow us to determine, again under administration of IL-6, the necessity of the IL-6-STAT3 pathway for the regulation of SERT as well as the effect on depression-related behaviour. Finally, to examine the relevance of this pathway in MIA, this model could be used as part of the Poly(I:C) paradigm, providing a powerful tool to investigate another aspect of SERT regulation on one hand, as well as an example of *Gene × Environment* interaction, specifically STAT3 and MIA, on the other hand.

To conclude, this study supports the notion that epigenetic mechanisms contribute to the environmental programming of brain development and behaviour by embedding the impact of the early life experiences on gene expression. Moreover, in association with the known behavioural effects, insight into molecular effects of MIA on the adult hippocampus is provided and suggests that a distinct hippocampal global and gene-specific histone acetylation pattern may contribute to embedding the impact of MIA on SERT expression and depression-like behaviour later in life.

6. ABBREVIATIONS

- BDNF: brain-derived neurotrophic factor

- CBT: cognitive behavioural therapy

- ChIP: chromatin immunoprecipitation

- CREB: cAMP response element-binding protein

- dsRNA: double-stranded RNA

- ECS: electroconvulsive seizures

- ECT: electroconvulsive therapy

- HAT: histone acetyl-transferase

- HDAC: histone deacetylase

- IFN: interferon (e.g. IFN-α: interferon-α)

- IL: interleukin (e.g. IL-6: interleukin-6)

- JAK: janus kinase

- KO: knock-out

- LPS: lipopolysaccharide

- LTP: long-term potentiation

- MDD: major depressive disorder

- MIA: maternal immune activation

- MAOI: monoamine oxidase inhibitor

- NMDA: N-methyl-D-aspartate

- NSAID: non-steroidal anti-inflammatory drug

- PAMP: pathogen-associated molecular patterns

- Poly(I:C): Polyinosinic:polycytidylic acid

- RT: room temperature

- SERT: serotonin transporter

- SNRI: selective norepinephrine reuptake inhibitor

- SSRI: selective serotonin reuptake inhibitor

- STAT: signal transducer and activator of transcription

- TCA: tricyclic antidepressant

- TLR: Toll-like receptor

- TMS: trancranial magnetic stimulation

- TNF-α: tumor necrosis factor α

- TRYCAT: tryptophan catabolite

- VEGF: vascular endothelial growth factor

- VEGFR: vascular endothelial growth factor receptor

7. BIBLIOGRAPHY

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87

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97

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8. ACKNOWLEDGEMENTS

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9. CURRICULUM VITAE

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Education

September 2012- Present

Master of Science (Molecular Biology)

University of Vienna, Austria

September 2007 - June 2011

Bachelor of Science (Biological Sciences)

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University of Edinburgh, UK

September 1995 - June 2007

School and High School (Examinations: Baccalaureat General, Matura)

Lycee Francais de Vienne, Austria

Research experience

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Master project: "Maternal immune activation epigenetically regulates
expression of the serotonin transporter in the adult offspring brain"
(Supervisor: Daniela Pollak)
Department of Neurophysiology and Neuropharmacology,
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February 2011 - March 2011

Bachelor project: "Innate fear of predators: An analysis of cerebellar c-fos
expression in mice exposed to trimethylthiazoline (TMT), a component of
fox faeces" (Supervisor: Ian Duguid)
Centre for Integrative Physiology
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June 2010 - July 2010

Internship: "The effect of cholesterol on the progression of Alzheimer-like
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Publications

The Poly(I:C)-induced maternal immune activation model in preclinical
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S Reisinger, D Khan, E Kong, A Berger, A Pollak, DD Pollak.
Pharmacology & Therapeutics [available online 4 January 2015]