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„THE ROLE OF MATERNAL CARE BEHAVIOR IN THE EFFECTS OF MATERNAL IMMUNE ACTIVATION ON DEPRESSION-LIKE BEHAVIOR IN THE MOUSE”

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Biology gives you a brain. Life turns it into a mind.

Jeffrey Eugenides, Middlesex

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1 Zusammenfassung

Depression ist eine der schwerwiegendsten psychischen Erkrankungen. Bereits im frühen Lebensalter stellt Stress einen spezifischen Risikofaktor dar, welcher die Wahrscheinlichkeit erhöht, an Depression zu erkranken. Es wurde bereits anhand eines Mausmodels gezeigt, dass pränataler Infektionsstress, welcher durch Immunstimulation der trächtigen Mausmutter (maternale Immunaktivierung, MIA) ausgelöst wird, mit depressionsähnlichem Verhalten und Veränderung der adulten hippokampalen Neurogenese der Nachkommen, im Erwachsenenalter, in Zusammenhang steht. Viele Psychopathologien rufen chronische Verhaltensveränderungen hervor, welche durch spezifische, epigenetische Modifikation der Genexpression kontrolliert werden. Diese epigenetischen Modifikationen werden oft in Zusammenhang mit frühen Lebensereignissen, wie beispielsweise mütterliches Fürsorgeverhalten (MF) bei Nagern und Menschen, gebracht. Ob genetisch bedingte Unterschiede in der MF Einfluss auf die ungünstigen frühen Lebensereignisse haben, welche das Verhalten der Nachkommen im Erwachsenenalter beeinflussen, wurde bisher noch nicht untersucht.

In der vorliegenden Studie wurden zwei ingezüchtete Mäusestämme, C57BL/6N (C57) und C3H/HeNCrl (C3H) verwendet, bei welchen stammspezifisch differentielles MF beschrieben wird. Anhand dieser beiden Stämme wurde experimentell untersucht, ob sich genetisch determinierte Unterschiede im MF feststellen lassen, welche das Ergebnis von MIA (depressionsähnliches Verhalten der erwachsenen Nachkommen) beeinflussen könnten.

MIA wurde durch systemische Applikation von Polyinosinic:Polycytidylic Acid (Poly (I:C); 20mg/kg) an trächtigen C57 und C3H Weibchen am embryonalen Tag 12.5 (E12.5) herbeigeführt. Das MF der Mütter gegenüber ihren Nachkommen wurde an den postnatalen Tagen 1 bis 6 aufgezeichnet und nach einem standardisierten Protokoll beurteilt. Depressionsartiges Verhalten der erwachsenen Nachkommen wurde anhand von anerkannten Verhaltenstests, wie dem Sucrose Preference Test (SPT) und dem Forced Swim Test (FST), evaluiert. Der mRNA-Gehalt der Glucocorticoid und

Mineralocorticoid Rezeptoren (GR und MR), welche bekannterweise durch MF reguliert werden können und auch an der Pathophysiologie von Depression beteiligt sind, wurden als mögliches molekulares Korrelat im hippokampalen Gewebe adulter MIA- und Kontroll-Nachkommen untersucht.

Es wurde in der vorliegenden Studie aufgezeigt, dass MIA das Nachkommen-orientierte Verhalten der MF signifikant verringert. Im Speziellen wird die Häufigkeit, mit welcher Mütter die Nachkommen durch das „Schleck-und-Putz“ Verhalten pflegen, signifikant reduziert. Dieses Versorgungsverhalten in der Pflege der Jungen wird als primäre Form von physischem Kontakt zwischen Mutter und Neugeborenen angesehen. Die Intensität dieses Kontaktes kann das Verhalten und die assoziierten autonomen und endokrinen Faktoren der Nachkommen im späteren Leben beeinflussen. Das gesteigerte depressionsähnliche Verhalten, welches durch MIA in adulten C57 Nachkommen gezeigt wurde, konnte bei C3H Mäusen nicht beobachtet werden. Als mögliches molekulares Korrelat dieser unterschiedlichen Empfänglichkeit für die Effekte der MIA auf depressionsähnliches Verhalten, könnte die MIA-induzierte Veränderung der mRNA Expression von GR oder MR im Hippokampus der adulten Nachkommen eine Rolle spielen.
Diese Beobachtungen zeigen, dass die genetische Variabilität zwischen C57 und C3H Mäusen den Effekt von MIA auf das depressionsähnliche Verhalten im späteren Leben beeinflusst, ohne stammspezifisch die unterdrückenden Effekte von MIA auf MF zu modulieren. Angesichts der durch MIA- und stammbeeinflussten hippokampalen Expression von MR und GR in adulten Nachkommen, gibt es mindestens zwei mögliche Interpretationen dieser Ergebnisse I.) das Verhalten im Erwachsenenalter, welches als Konsequenz von MIA gilt, wird nicht von MF beeinflusst; II.) die genetisch festgelegte, stammabhängig unterschiedliche Empfänglichkeit für die Genexpression-regulierenden Effekte von MIA in Bezug auf GR und MR im Hippokampus, dient als molekularer „Puffer-Mechanismus“, um die nachteiligen Folgen der von MIA induzierten Störung der MF in C3H Mäusen zu dämpfen.

Die vorliegende Studie stellt die Grundlage für zukünftige Experimente, wie zB. Fremdenpflege (Cross-Fostering) dar, mit deren Hilfe man den Einfluss der MIA-

induzierten Störung der MF auf systemischer und molekularer Ebene weiter untersuchen könnte, um die genetische Grundlage, für die Empfänglichkeit bzw. Widerstandsfähigkeit für MIA-assoziierte Modulation im depressionsähnlichen Verhalten im späteren Leben, aufzuklären.

2 Abstract

Mood disorders, including major depression, are some of the most devastating mental illnesses. Early life stress is known as a risk factor for the development of several psychiatric disorders, including depression. Prenatal infectious stress, induced by maternal immune activation (MIA), has been found to be associated with depression-like behavior and alterations in adult hippocampal neurogenesis later in life in a mouse model. Long-lasting behavioral adaptations, including those related to psychopathologies, can be controlled by specific epigenetic modifications, which are subject to be influenced by early life events, including maternal care behavior (MB). Whether genetic differences in MB can moderate the impact of adverse early life events on emotional disturbances later in life, has not been investigated so far.

In our study we employed two inbred mouse strains, which are reported to represent distinctive MB, to examine whether differential levels of pup-orientated MB modulates the effect of MIA on depression-like behavior in adult offspring.

MIA was induced by the administration of polyinosinic:polycytidylic acid (Poly (I:C)) (20 mg/kg, i.p.) at embryonic day 12.5 (E12.5) to pregnant C57BL/6N (C57) and C3H/HeNCrl (C3H) mice and maternal behavior was recorded on the postnatal days 1 to 6. Depression-like behavior of adult offspring was evaluated using standard behavioral tests, including the Sucrose Preference Test (SPT) and the Forced Swim Test (FST). mRNA levels of glucocorticoid and mineralocorticoid receptors (GR and MR), known to be subject to the regulation by MB and implicated in the pathophysiology of depression, were evaluated as potential molecular correlates in hippocampal tissue of C3H and C57 offspring.

MIA significantly reduced the percentage of time mothers of both strains engage in pup-orientated behaviors, specifically the frequency of licking/grooming, the main form of physical contact experienced by the newborn pups, which is known to modulate behavioral, autonomous and endocrine functions later in life. However, the enhancement

in depression-like behavior resulting from MIA in adult C57 offspring was not observed in C3H mice. The possible molecular correlate between MB and depression-like behavior has shown that the hippocampal corticosteroid receptor expression is modulated in adult C57 and C3H offspring by different factors, like sex, strain and MIA-treatment.

We here firstly describe the significantly dampening effect of Poly(I:C)-induced MIA on maternal behavior in the mouse. Interestingly, although MIA suppressed MB in both C57 and C3H strains, the modulation of depression-like behavior resulting from MIA previously reported for C57 mice, was not detectable in adult C3H offspring. These observations provide evidence that the genetic background moderates the effect of MIA on depression-like behavior later in life without altering its impact on MB. Considering that MIA strain-dependently regulated hippocampal expression of GR and MR these results allow for two possible conclusions: i.) MB is not mediating the behavioral consequences of MIA in adulthood; ii.) the differential genetically-determined molecular variations susceptibility of C3H versus C57 mice to the MIA-induced regulation of GR and MR expression in the adult offspring hippocampus provides a molecular buffer mechanism dampening the adverse consequences of MIA-induced disruption of MB in C3H mice.

The present study hence invites future investigations, including cross-fostering experiments, aiming to determine the definite causal involvement of deranged MB in the effects of MIA on depression-like behavior later in life and potentially elucidating the genetic basis for susceptibility/resilience to the impact of this form of adverse early life events on the regulation of emotional behavior.

3 Introduction

3.1 Depression

Major depressive disorder (MDD) is a widespread mental disease with about 15% of all people worldwide experiencing the associated symptoms at some point in life (Bromet et al. 2011). The features of this psychiatric disease are determined by the sustained presence of distinct symptoms including emotional, psychological and somatic ones. These symptoms encompass depressed mood, anhedonia, fatigue, sleep disturbances, cognitive dysfunctions and suicidal ideation (Holtzheimer & Mayberg 2011).

MDD not only impairs the quality of a persons' life but also represents a substantial burden on the world's population (Whiteford et al. 2013). Considering the mortality and disability of all disease burdens, depression is currently ranked 11th worldwide. Especially in highly developed countries, MDD is one of the fifth leading disease burdens (Murray et al. 2012). Furthermore, the incidence of MDD is permanently increasing by 38% between 1990 and 2010 and this trend is expected to last until major progresses in treatment and disease management can be achieved (Murray et al. 2012).

To date, a number of drug treatment approaches exist, including selective serotonin or norepinephrine reuptake inhibitors (SSRIs/SNRIs), tricyclic antidepressants and monoamine oxidase inhibitors (Davidson 2010; Levinstein & Samuels 2014). However, similar to other neuropsychiatric disorders, major depression has many limitations regarding available treatments.

Currently available pharmacological antidepressants are mainly targeting molecular mechanisms of monoaminergic neurotransmission and albeit the fact that they do serve to alleviate symptoms associated with depression in some patients, there are a several problems related to their usage. One common concern is the delay of onset in terms of clinically relevant effects, a poorly understood phenomenon (because it cannot be completely described by the current knowledge about the activity at the monoaminergic synapse), which negatively impacts patients´ compliance (Levinstein & Samuels 2014). Moreover, drug treatment is often completely ineffective in a large percentage of patients; e.g. Citalopram, one frequently prescribed antidepressant has been shown to

achieve its effect only in 30% of patients in a large clinical trial (Trivedi et al. 2006). Even in patients, who were treated with four different, well-known antidepressants, only about one third were experiencing a reduction in depressive symptoms, and about 20% of all patients are not responding to any existing treatment and therefore are termed as “treatment resistant” (Rush et al. 2006; Holtzheimer & Mayberg 2011). On top of that, a considerable percentage of patients are faced with a series of adverse side effects, including impacts on the cardiovascular system, weight gain, nausea, sleep disturbances and several others, which may significantly affects an individuals’ quality of life (Whiskey & Taylor 2013; Gartlehner et al. 2008).

There are also non-pharmacological approaches available for the treatment of MDD patients, like psychological interventions, including cognitive behavioral therapy (CBT) and interpersonal psychotherapy. These treatments are in some cases believed to be as effective as antidepressant drugs (Picardi & Gaetano 2014). However, it has been proposed that the combination of psychosocial and pharmacological therapy may present the best treatment for severe and chronic depression (Hollon et al. 2014). Further non-pharmacological approaches, include the recently emerging transcranial magnetic stimulation therapy (deep TMS) (Morishita et al. 2014) and the electroconvulsive therapy (ECT), are often used in “treatment resistant”-patients (Group 2003; Khalid et al. 2008).

Although, there are many different therapeutic strategies available to treat MDD, the described limitations are largely considered due to their mode of action not being based upon interventions related to the cellular and molecular mechanisms of the disease. This problem relates to the still not fully understood disease principles of major depression, which are consequence of the complexity and the heterogeneity of the disease. Several hypotheses regarding the pathophysiology of MDD are currently being considered, some of them will be outlined in the following sections.

3.2 Hypotheses of Depression

The best-known hypothesis of depression is the *monoamine hypothesis*. The theory assumes that depression is due to the dysbalanced monoamine levels in the brain. Trails confirmed that the action of commonly used antidepressant drugs (e.g. SSRIs) was very effective in the serotonergic system (Krishnan & Nestler 2008). This impact on serotonergic neurotransmission system is assumed to restore the balance of the serotonin system (Lee et al. 2010), a major regulatory circuitry controlling mood and emotional behavior (Lee et al. 2010; Haase & Brown 2014).

Ongoing research about the molecular pathways of MDD has yielded a high variation of other hypotheses about the pathogenesis of this mood disorder and only a single theory may never be suitable to explain such a complex disease (Krishnan & Nestler 2008). Due to the large number of people who are diagnosed with depression and the limitations of the physiological markers accessible, it is likely that the complexity of the symptoms of depression belong to several distinct pathophysiological mechanisms rather than to one definite disease.

The following paragraph describes another specific theory of depression which is well supported by a rising number of publications in recent years and which importantly relates to the rationale for the present project: the *immune hypothesis of depression*.

3.2.1 Immune Hypothesis of Depression

The immune system is thought to play a major role in the development and progression of depressive disorders (Anisman et al. 2005; Hayley et al. 2005).

Several seminal studies investigated the link between MDD and inflammatory states, especially after observing a connection between symptoms revealed by depressed patients and the experimentally induced “sickness behavior” in animals. Features displayed by subjects with sickness behavior, like lethargy, anhedonia and reduced mood, are similar to the somatic and psychological representations resulting from an infection with a pathogen and related to symptoms observed in depressed patients (Dantzer et al. 2008). It is considered that the activation of pro-inflammatory cytokines, including interleukin-1β (IL-1β), interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α)

mostly relates to the observed symptoms. While these cytokines are in general protective and they do have physiological roles in the CNS, such as trophic support of neurons (Anisman et al. 2005; Hayley et al. 2005), their effects can become maladaptive if the inflammatory state is extended in magnitude and/or time (Dantzer et al. 2008). The link between MDD and increased plasma levels of proinflammatory cytokines such as IL-6 and TNF-α was supported by a number of meta-analysis from patient studies (Dowlati et al. 2010; Valkanova et al. 2013; Howren et al. 2009). Additionally, another report demonstrates a modulation of plasma levels of cytokines by SSRI-treatment, including TNF-α, IL-6 and IL-1β (Hannestad et al. 2011).

However, the connection between inflammatory mechanisms in the periphery and changes in the central nervous system (CNS) are not yet entirely elucidated (Miller et al. 2009) and also the pathomechanistic relevance of augmented inflammatory states in depression, are still incompletely understood. Several hypotheses exist, a prominent one relates to the regulation of serotonin levels as a result of immune activation. Here, it is assumed that certain cytokines activate indoleamine 2,3-dioxygenase (IDO) which catabolizes the reaction of tryptophan into tryptophan catabolites (TRYCATs). Tryptophan is the precursor of serotonin, and this above described reaction limits the availability of tryptophan for the synthesis of serotonin, consequently decreasing the serotonin levels systemically (Maes 2011; Maes et al. 2011). Additionally, some TRYCATs have also neurotoxic, depressogenic and anxiogenic effects (Maes et al. 2011; Maes 2011; Wichers et al. 2005). Moreover, activation of the IDO-pathway has been shown to result in decreased adult hippocampal neurogenesis, which is importantly and strongly associated with depression and its presumed pathomechanism (Anderson et al. 2013).

In conclusion, the molecular mechanism of cytokines, contributing to depression, is suggested to be the result of an activated immune response, where the pro-inflammatory cytokine production increases, resulting in adverse effects on neuronal (Miller et al. 2009) function in brain regions, which are involved in the neural circuitry of emotional regulation. This molecular mechanism has also been shown to be prevented by blockade of CNS cytokines with certain inhibitors with proposed antidepressant

effects (or the promotion of anti-inflammatory cytokines) (Anisman et al. 2005; Hayley et al. 2005).

Relating and interacting with the immune hypothesis of depression, another theory which is strongly supported by numerous lines of evidence from scientific literature will be described in the next section, as it importantly relates to the present project: *the stress hypothesis of depression*.

3.2.2 Stress Hypothesis of Depression

People who experience chronic stress in their daily life have a higher risk of developing depression, hence stress is thought to be a precipitating factor for the development of the disease (Hill et al. 2012). This continuous stress exposure induces changes in intracellular signaling pathways, gene expressions, cellular morphologies and neuronal functions of brain regions which are part of the neural circuitry involved in depression and these changes mainly result from the release of corticosteroids. These brain areas, including the hippocampus and the medial prefrontal cortex (mPFC), are the ones mainly involved in mood control, cognition and executive function (Mineur et al. 2003).

Long-term consequences of chronic exposure to elevated levels of corticosteroids are morphological changes in the brain which may lead to functional deficiencies due to neuronal atrophy. Indeed, it has also been demonstrated that there is a reduction of hippocampal volume and also the adult neurogenesis in the hippocampus is decreased in depressed patients and chronic stress-based animal models of the disease (Strekalova et al. 2004; Duric & Duman 2013).

The reaction of an individual to a stressful event activates a variety of behavioral and physiological responses, collectively termed “stress response” (Smith & Vale 2006). This stress response is initiated in the central nervous system and executed through effectors in the periphery, forming part of the hypothalamic-pituitary adrenal (HPA) axis. The major structures of the HPA axis comprise the paraventricular nucleus (PVN) of the hypothalamus, the anterior lobe of the pituitary gland and the adrenal gland (Habib et al. 2001; Chrousos 1992; Whitnall 1993). The hypophysiotropic neurons of the PVN synthesize and release the major regulator molecule, the corticotropin releasing

hormone (CRH), which is modulating the HPA axis response (Vale et al. 1981; Rivier & Vale 1983). As a reaction to stress exposure, CRH is secreted to the hypophysial portal vessels of the anterior pituitary gland where it binds to its respective receptors inducing the release of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH then binds to ACTH receptors in the adrenal cortex, hereby triggering glucocorticoid synthesis and release. Thus, glucocorticoids are the downstream effectors of the HPA axis which regulate physiological and behavioral mechanisms (Munck et al. 1984; Bamberger et al. 1996) related to stress exposure. The HPA axis functions as a feedback loop to sustain a balance in the levels of circulating glucocorticoids, which comprise corticosterone, the main glucocorticoid in animals and cortisol, the most important glucocorticoid in humans (de Kloet 2014). While the biological responses induced by the action of glucocorticoids are adaptive, chronic over- activation of the HPA axis may cause pathologies (McEwen & Stellar 1993).

The feedback loop of the HPA axis is controlled by glucocorticoid receptors (GRs) which are widely distributed in the brain. The GR modifies the transcription of stress-responsive brain regions like HPA components (Reul & de Kloet 1986).
Briefly, the feedback inhibition of the HPA axis comes from circulating glucocorticoids (Keller-Wood & Dallman 1984)**.** GRs and Mineralocorticoid Receptors (MRs) have different affinities for glucocorticoids. MR controls the basic HPA tone and GR modifies the glucocorticoid negative feedback loop following stress (E. R. De Kloet et al. 1998; Reul & de Kloet 1986; Ratka et al. 1989). Furthermore the PVN and the hippocampus are key structures for the glucocorticoid feedback inhibition of the HPA axis. High levels of GR and MR and infusion of glucocorticoids in these areas reduce the PVN neuronal activity, increase the adrenalectomy-induced ACTH hypersecretion and decrease basal- and stress-induced glucocorticoid release in the hippocampus (Smith & Vale 2006).

In this regard, one of the well-validated findings is a consistent association between alterations in the HPA axis and depression, i.e. dysregulation of the HPA axis has been proposed as a central feature relating to the pathogenesis of MDD (Peeters et al. 2004; Pariante et al. 1995). In depressed patients, the physiological activity of the HPA axis has been found to be impaired resulting in an aberrant secretion of cortisol. Such

impairment is also reflected in abnormal periodicity in the HPA axis activity which is one major criterion for a disturbed biological system (Siever & Davis 1985).

Interestingly, the cellular and molecular mediators of the stress response also interact with key players which are involved in the activation of the immune system. As such, the release of CRH leads to a secretion of catecholamines and adrenocorticotropic hormones which enhance the NFkB-DNA binding in macrophages and these produce and deliver proinflammatory cytokines. Those cytokines, including IL-1β, TNF-α induce an inflammatory signaling cascade leading to changes in the metabolism of the monoamines and neurotrophic support in the brain (Miller et al. 2009).

Under physiological conditions, the cytokine-induced activation of CRH and the subsequent cortisol release suppress the NFkB activation, reducing the inflammation and respectively terminating the immune response. Chronic stress results in a reduced sensitivity of the corticosteroid receptors and disrupts the feedback loop resulting in a dysbalance between pro-and anti-inflammatory responses (Miller et al. 2009).

Corticosteroid Receptors

The corticosteroid receptors, central to effects of glucocorticoids, mediating the biological impact of HPA axis regulation and dysregulation, are also importantly linked to pathomechanism of depression and are examined in the present project. Therefore, their characteristics and relevance will be explained in more detail in the following section.

The steroid receptor superfamily comprises receptors for steroids which are found on the plasma membrane, in the cytosol and in the nucleus of target cells. The binding of a steroid hormone to its receptor initializes a signaling cascade which results in alteration of gene expression (Lu et al. 2006; Kumar & Thompson 1999). Most corticosteroid receptors are members of the nuclear receptor subfamily, encompassing GR and MR. Major ligands for those receptors are corticosterone (CORT) in most animals, including rodents, and cortisol in humans (Gourley & Taylor 2009). These steroid hormones are produced in the adrenal gland and are involved in the regulation of energy, immune reaction and stress response. They can enter the brain by passing the blood-brain-barrier and affect those cells containing GRs and MRs hereby influencing

neuroendocrine function, cognition, neurogenesis, neurodegeneration and cell survival in the CNS (Rozeboom et al. 2007).

The expressional levels of GR and MR are particularly high in pyramidal and granular neurons of the limbic structures, including the hippocampus and the frontal cortex (Joëls et al. 2013). As laid out before, a proper stress response is important for adaption to real threats and increased glucocorticoid levels are necessary for restructuring of energy resources. However, chronic stress and constant elevation of glucocorticoids can last in long-term changes, impairing the neuronal hippocampal function, which relates to dysregulation of the HPA axis and several psychopathologies (Conrad 2008), such as depression (Beck-Friis et al. 1985). Additionally, aberrant GR expression itself is also associated with mood disorders (Matsubara et al. 2006) and an altered amount of GRs in certain brain regions may make those subjects more susceptible to the toxic effects of excessive glucocorticoid levels (M J Webster, M B Knable, J O'Grady, J Orthmann and C S Weickert 2002). Experience of chronic stress also decreases the density of MR in the hippocampus, resulting in a compensatory increase in the activity of HPA axis (Gesing et al. 2001).

There is ample evidence that stress exposure and the elevated immune activity may not only shape existing neuronal responses at the molecular, cellular and systemic levels, as described above, but may also affect prenatal development, especially the brain hereby contributing to an enhanced susceptibility for the development of mental illnesses, including mood disorders later in life (Mineur et al. 2003; Hayley et al. 2005; McEwen 2003). The investigation of this developmental effect of maternal immune activation and its association with depression is at the core of the present research project.

Early Life Stress

Early adverse life events, like stress exposure during the gestational period and traumas in the childhood period can alter the sensitivity to the effect of stress later in life and affect an individual's vulnerability to depression (McEwen 2003).

Maternal Psychosocial Stress

The experience of prenatal stress has been demonstrated to have a deleterious impact on the development of the fetal brain associated with an enhanced susceptibility for the development of psychiatric disorders later in life (Fine et al. 2014). The augmented exposure of the female to stressful events during pregnancy, through endocrine and autonomous routes, may alter conditions of the fetal environment hereby contributing to the modulation of the “fetal programming”, determining brain development and gene x environment interactions later in life. While there are difficulties in defining a concrete definition of maternal stress in human pregnancy and the complexity of the various components of stressful experiences hamper the comparability of individual cases, several animal models of early life stress exist. These animal paradigms allow further investigations of the long-term effects of maternal stress, on neuronal function and development of neuropsychiatric diseases, under controlled conditions. E.g. using the prenatal restraint stress (PRS) paradigm as a form of maternal psychosocial stress (Morley-Fletcher et al. 2003; Maccari & Morley-Fletcher 2007) a sustained impact of early gestational stress on the HPA axis, together with an exacerbated stress response in the offspring, has been demonstrated (Maccari et al. 2003).This hyperactivity of the HPA axis has been further shown to increase the anxiety- and depressive-like behavior (Morley-Fletcher et al. 2003), along with an impairment in the proper activation of the innate immune response in adult PRS animals (Maccari & Morley-Fletcher 2007).

Maternal Infectious Stress

Strong experimental evidence resulting from epidemiological studies in the human population and increasing data obtained from research in experimental animals suggest maternal infectious stress as another form of early life stress negatively impacting fetal development. It is also associated with an enhanced risk for various psychiatric disorders later in life (Meyer et al. 2007; Khan et al. 2014; Reisinger et al. 2015; Brown 2012; Bitanihirwe et al. 2010; Boksa 2010; Meyer & Feldon 2009; Meyer 2014).

The mechanism underlying gestational infectious stress is highly complex, because at the same time the maternal immune system has to prevent rejection of the fetus itself and has to sustain defense against intruding pathogens (Riley & Nelson 2010).

For a healthy fetal developmental process a certain immune balance, between the maternal and the fetal environment, is needed (Deverman & Patterson 2009). The interaction of the immune system, between the maternal and fetal compartment, is provided by the structural interface, termed placenta (Mehler & Kessler 1998; Garay et al. 2013). The hemochorial placenta, of both humans and rodents, allows direct contact between fetal an maternal part (Colucci et al. 2011). Under physiological conditions, the process between the maternal and fetal compartment is tightly controlled by the equilibrium of cytokines (important signaling molecules which are part of the innate immune system and regulators of the development) (Garay et al. 2013; Deverman & Patterson 2009). However, maternal infection leads to cytokine imbalance which has molecular, structural and functional consequences for fetal brain and can result in long-lasting effects, such as the susceptibility for the development of a neuropsychiatric disorder in adulthood (Patterson 2007; Meyer & Feldon 2009).

However, the causality and molecular mechanisms underlying the link between maternal immune activation (MIA) and MDD are nearly impossible to investigate in human population. Therefore animal models of MIA present an important instrument in studying the mediating pathophysiological mechanisms. To this end, the present study is employing a particular model of MIA based upon the use of Poly (I:C), a synthetic viral analogue, for the induction of MIA in mice.

3.3 Animal Models

Animal models are used for mimicking mental diseases existing in humans, hereby helping to increase the understanding of the neurobiological basis underlying psychiatric illnesses and to find novel therapeutic targets (Krishnan & Nestler 2008; Meyer 2014). However, natural limitations to the use of animal models exist, considering the complexity of neuropsychiatric disorders and the uniqueness of some associated symptoms (e.g. thoughts of suicide, delusions, etc.) of humans. Hence, without anthropomorphizing animals and their behavior, one should concentrate on modeling the underlying mechanisms which are observed in mental illness-related behavioral phenotypes and accompanying endophenotypes. These mechanisms include changes in the related neurotransmitter systems, brain structures and gene expression (Hall et al. 2014). Thus, rather than aiming to model a complex mental illness in its entirety in experimental animals, it may be more productive to concentrate on the reproduction of certain features (Meyer & Feldon 2012). These animal models offer a priceless instrument to study the causal mechanisms underlying mood disorders and permit experimental manipulations that are not possible in human studies, which are only based upon observation of symptoms, imaging techniques and analysis of postmortem brain tissue (Pollak et al. 2010; Zipursky 2007).

3.3.1 Animal Models of Gestational Infection

Two major animal models for the investigation of the pathophysiological relevance of maternal gestational infectious stress exist: one, based upon the application of the bacterial endotoxin Lipopolysaccharide (LPS) and the other one employing a viral analogue called Polyinosinic:Polycytidylic acid (Poly (I:C)). Both of them are used for inducing an immune activation of the pregnant dam which is thought to affect the proper development of the fetal nervous system (Meyer & Feldon 2009), and can cause neurobehavioral deficits later in adulthood through mechanisms of cytokine induction, as described in the previous sections.

Lipopolysaccharide

Exposure to LPS, a cell wall component of gram negative bacteria, induces activation of the Toll-like receptor 4 (TLR-4) signaling pathway. Administration of LPS to experimental animals induces the experience of sickness behavior. Further, MIA resulting from LPS administration is linked to anxiety and depression-like behavior in adult offspring (Bernardi et al. 2014).

LPS has an influence on metabolic, neuroendocrine and behavioral factors which includes the reproductive development, growth, puberty onset, estrous cycle, sympathetic nervous system, HPA axis and hypothalamic-pituitary gonadal (HPG) axis activity, and has a greater effect on females than on males (Markham & Koenig 2011; Walker et al. 2012).

LPS-treatment in pregnancy has been also associated with a reduction in maternal care resulting in increased stress-related behaviors and neuroendocrine perturbation of the pups. Interestingly, a transgenerational passage of this effect, presumably based upon epigenetic modification in the maternal but not the paternal line, has been observed (Walker et al. 2010; Walker et al. 2009). As such, neonatal LPS exposure increases the risk of anxiety-like behavior in adulthood and impairs maternal care, which is passed on to the next generation, consequently again modulating the long-term stress response and behavior of second generation (Liu et al. 1997; Shanks et al. 2000; Walker et al. 2012; Walker et al. 2010; Walker et al. 2009).

Poly (I:C)

Poly (I:C), containing one polymer strand with inosinic acid and another with cytidylic acid, is a dsRNA analogue and mimics viral pathogens (Tatematsu et al. 2014). The viral dsRNA represents the viral genome and emerges from the replication of RNA or DNA viruses in the host animal (Tatematsu et al. 2014). Both, dsRNA and Poly (I:C) bind to the Toll-like receptor 3 (TLR-3), hereby initializing an innate immune response cascade (Perales-Linares & Navas-Martin 2013). The activation of TLR-3 triggers a signal transduction event ultimately leading to upregulation of anti-viral, pro-inflammatory and pro-apoptotic factors, mainly through activation and nuclear translocation of NFkB (Meyer 2014; Bitanihirwe et al. 2010; Riley & Nelson 2010; Perales-Linares & Navas-

Martin 2013). This immune response to a viral pathogen, where production of pro-inflammatory cytokines takes place, may be harmful to the development of the fetus (Garay et al. 2013; Deverman & Patterson 2009).

As mentioned above, Poly (I:C) is an immune stimulating component which is used for MIA rodent models through its systemic administration to pregnant dams. The Poly (I:C) MIA model has been shown to be well fitted to imitate the effect of maternal viral infection and its subsequent cytokine response and impact on fetal development. Albeit the fact, that stimulated animals do not display the whole immune response which would be initiated by a natural pathogen (Meyer & Feldon 2009). However, the experimental manipulation during the developmental process allows to closely mimic the situation in the human population where environmental interferences during pregnancy are considered as major cause for the increased likelihood to develop psychiatric disorders in adulthood (Powell 2010; Meyer & Feldon 2009).

Consequently, important insights into the pathophysiology of mental illness have been obtained using the Poly (I:C) MIA model (Garay et al. 2013; Patterson 2007; Deverman & Patterson 2009; Meyer 2014; Meyer & Feldon 2012; Meyer et al. 2007; Gibney et al. 2013; Khan et al. 2014; Meyer et al. 2006; Reisinger et al. 2015).

Specifically, with regards to depression-like behavior, our group has recently shown that Poly (I:C) administration to pregnant mice at embryonic day 12.5 (E12.5) induces depression-like behavior in adult offspring which is accompanied by neurogenic and neurotrophic deficits (Khan et al. 2014).

However, it remains unknown, how prenatal infectious stress resulting from gestational Poly (I:C)-based MIA interacts with other perinatal adversities, such as alterations in maternal care behavior (MB) and genetic factors, leading to emotional disturbances later in life.

3.4 Maternal Care Behavior

The development of the neuronal system and the resulting functions, including behavioral aspects, depend on the intricate interaction of genes with environmental factors. Early life experiences, like stress or parental care, may determine the sensitivity of an individual to psychiatric disorders in adulthood (Koehl et al. 2012). The maternal care behavior itself, as important determinant of an individual's emotional phenotype later in life (Crabbe et al. 1999; Wahlsten et al. 2003), is subject of modulation by both genetic and environmental factors.

Postnatal interferences (like handling, maternal separation, environmental enrichment or cross-fostering) affect the quality and quantity of MB, ultimately influencing the offspring's brain morphology and behavior in adulthood (van der Veen et al. 2008).

In a study, the impact of the genetic influence on MB (referring to inter- and intrastrain cross-fostering) of C57 and DBA mice (two common laboratory mouse-strains) has been found to be comparable regarding their biological pups. Two other common laboratory mouse strains, the AKR and C3H strain, display significant differences in MB, with AKR being less pup-oriented and C3H dams presenting with high levels of MB. Interestingly, the genetic background of the fostered pups influences MB (van der Veen et al. 2008) and pups benefit more from a mother of the same strain than from a mother of a different strain (Hager & Johnstone 2003). This finding could be due to the differences in the quality of ultrasonic vocalization of pups where distinct strains differ in their frequency range (Cohen-Salmon et al. 1985).

In addition, various mice strains differ in the morphology of the preoptic nucleus which is a main area controlling MB (Numan 2007). Distinct strains showed different corticosterone responses to stress (Cabib et al. 1990; Jones et al. 1998; Shanks et al. 1990) where glucocorticoid receptor expression increases licking of pups from dams (Rees et al. 2004).

In rodent models, the quality of MB is mainly evaluated by the frequency of pup licking and grooming. Licking/grooming (LG) is the major source of tactile stimulation for neonatal rodents which influences endocrine, cardiovascular, behavioral systems (Vialou et al. 2013), somatic growth and neural development (Liu et al. 2000). High

levels of MB raises the hippocampal levels of NMDA receptors (glutamate receptor), which lead to enhanced expression of brain-derived neurotrophic factors (BDNF), and hippocampal synaptogenesis. Therefore the long-term potentiation for learning and memory in the hippocampus is increased in adulthood (Liu et al. 2000). Contrary, maternal separation reduces growth-hormone release and enhances adrenal glucocorticoid levels which attenuate the expression of BDNF.

Moreover, the quality of the postnatal maternal environment (high and low level of MB) and the genetic background of an individual have a major effect on adult hippocampal neurogenesis, which is negatively associated with stress, anxiety and depression and positively linked to learning (Koehl et al. 2012). The amount of MB specifically influences the differentiation of immature neurons. High levels of MB are related to decreased anxiety, depression and stress response and to increased learning abilities (Koehl et al. 2012). Furthermore MB is considered as a major determinant for later life regulation of emotional, cognitive and neuroendocrine responses to stress (Danielle L. Champagne et al. 2008).

Interestingly, the specifics of MB and its modulation by external factors can be transmitted over generations, as recently shown in assessing the effects of neonatal immune stimulation by LPS (Walker et al. 2012). Here the F2 generation, originating from the mating of LPS- treated males with untreated females, showed no different effect of MB. In contrast, the F2 females from F1 LPS treated dams spent less time in LG behavior compared to saline dams (Walker et al. 2012). In parallel, the augmented anxiety-like phenotype in adulthood was also found to be transmitted across generations and maternal care could be the transgenerational mediator of this effect, possibly via epigenetic modifications along the maternal line (Liu et al. 1997; Shanks et al. 2000; Walker et al. 2009; Walker et al. 2012)

3.4.1 Long-Term Effects of Maternal Care

In the search for the biological mechanisms underlying life-long and even transgenerational effects mediating the impact of MB on behavior, stress and disease susceptibility in adulthood, epigenetic processes, appear to be a likely candidate

because they can be dynamically modulated by environmental factors (e.g. MDD) (Champagne 2013). Indeed, epigenetic mechanisms, which constitute an enduring regulatory impact on gene expression, which does not involve alterations in the sequence of the DNA, has been highly implicated as molecular interface of gene x environment interactions, jointly shaping brain development and neural functions in adulthood (Figure 1). Both of the two best studied events, DNA methylation and post-translational histone modification, have been extensively associated with the impact of early life events, including maternal care and subsequent long-lasting behavioral alterations (Kundakovic & Champagne 2015). Additionally, strong evidence for a relevance of epigenetic processes in the pathophysiology of several psychiatric disorders, including depression and their involvement in the biological mechanisms mediating the responses to psychopharmacological treatments, exist (Vialou et al. 2013; Krishnan & Nestler 2008; Anderson et al. 2013).

Figure 1

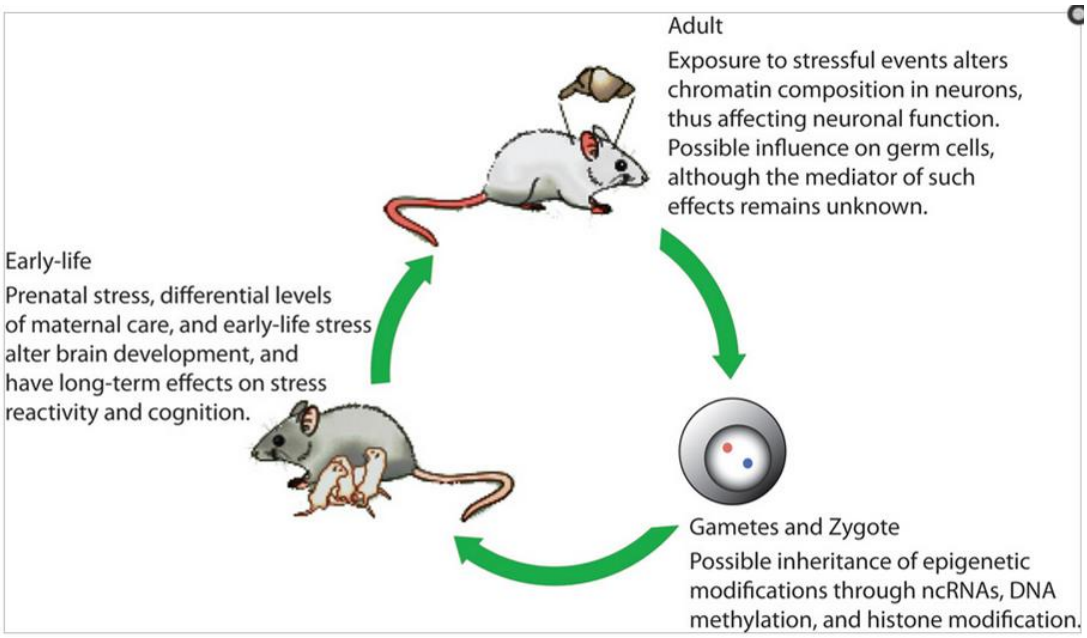

Figure 1. Epigenetic modifications in life span.

Early life events like a prenatal stress or changes in maternal care alter the brain development and can result in long-term consequences like altered stress responsiveness and cognition. Moreover stress exposure at adult animals affects neuronal function by changes in chromatin arrangement. There has to be a potential inheritance of epigenetic modifications through RNAs, DNAs, methylation and histone modification (Image source: Vialou et al. 2013).

With regards to MB in rodents, it has been demonstrated that female offspring display similar LG behavior which they themselves were exposed to in their early postnatal period. MB is thought to constitute a matrilineal transmittable behavior that is mediated by long-term changes in oxytocin levels in the hypothalamic medial preoptic area and the subsequent regulation by estrogen. The expression of estrogen receptor α (ERα) is reduced in low LG dams, since methylation at its gene promoter is increased, which decreases the ability of estrogen to activate the expression of the receptor for oxytocin (a key hormone regulating social bonding and MB) (Champagne et al. 2006; Champagne 2008). In comparison, the high LG animals have decreased methylation at the ERα promoter, increased ERα expression, as well as an increased estrogen sensitivity, which leads to a better oxytocin function and results in high LG behavior (Champagne & Meaney 2007). The epigenetically modulation of ERα expression arises during early postnatal weeks and preserves into adulthood. These long-term epigenetic effects of maternal care can be passed from one generation to the next which only affect the pups' brain without altering germ cells (Vialou et al. 2013).

At the behavioral levels, it has been shown that pups from high LG dams have lower levels of methyl marks and the animal is less sensitive to stress, than pups reared by mothers with low LG behavior where the genes gain methyl marks and pups grow up to be more stress responsive. Pups with "better mothers" will be more attentive towards their own pups and offspring which experienced low maternal care display lower LG behavior themselves (Figure 2) (Vialou et al. 2013; Nestler 2011; Weaver, Cervoni, et al. 2004).

Figure 2

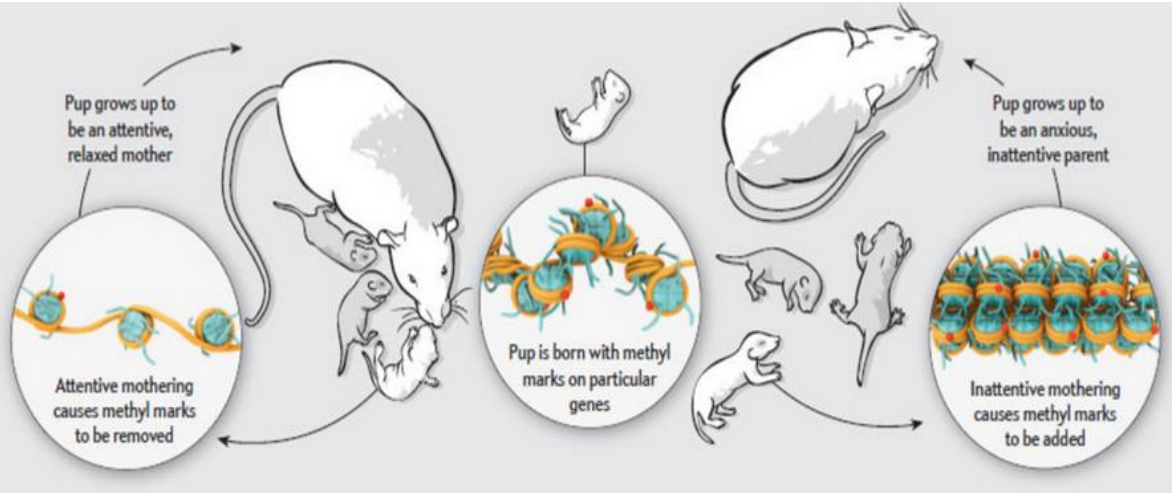

Figure 2. Long-term epigenetic mechanism of maternal behavior.

These genes which are involved in controlling stress responses and maternal behavior are differentially methylated. The more attentive the mother, the less methyl marks are on these genes and the pup itself grows to be an attentive mother. When pups are reared from a mother who is inattentive, methyl marks are added and the pup grows up to be an anxious parent itself (Image source: Vialou et al. 2013).

However, lower MB is not always negative, because rodents which experienced low LG display an enhanced response to stress but also contextual learning is better under stressful events in comparison to high LG pups (Bagot et al. 2009; Champagne 2008). In conclusion, MB causes a long term behavior adaption which raises the stress responsiveness and makes the offspring ready to survive in a more harmful environment (Vialou et al. 2013).

One likely candidate gene, undergoing epigenetic modification as a result of the quality of MB and implicated in the behavioral outcome, including pathological alterations in the offspring, is the glucocorticoid receptor. It has been demonstrated that handling of rats in the early postnatal days resulted in a higher pup-directed MB later in adulthood (Champagne 2013). The handling effect mimicked a maternal-infant interaction and increased the protein levels of GRs in the hippocampus and the frontal cortex in rodent brains and reduced the HPA response to stress (Liu et al. 1997). More handling or higher levels of maternal care, especially LG, modified the HPA response and induced transcription of genes encoding for GR. Furthermore, handling of pups increased the neurogenesis in the DG of the hippocampus and the neocortex in comparison to non-handled offspring (Joseph Altman 2004).

In addition, handling had an effect on plasma thyroid hormone levels which were increased in handled pups. By suppression of the thyroid synthesis, the outcome of handling on the hippocampal GR concentration (independent of plasma corticosterone levels) was blocked, suggesting an intermediate pathway between thyroid signaling and GR levels (Meaney et al. 1987). Interestingly, serotonin levels in hippocampal neurons are affected by handling and thyroid hormones and serotonin itself have an influence on hippocampal GR (Mitchell et al. 1990). Handling and thyroid hormones rise serotonin in neurons and if serotonin receptors get blocked, the serotonin induced increase in GR is suppressed and blocks therefore the outcome of handling (Figure 3) (Weaver, Diorio, et al. 2004; Meaney et al. 1993).

Figure 3

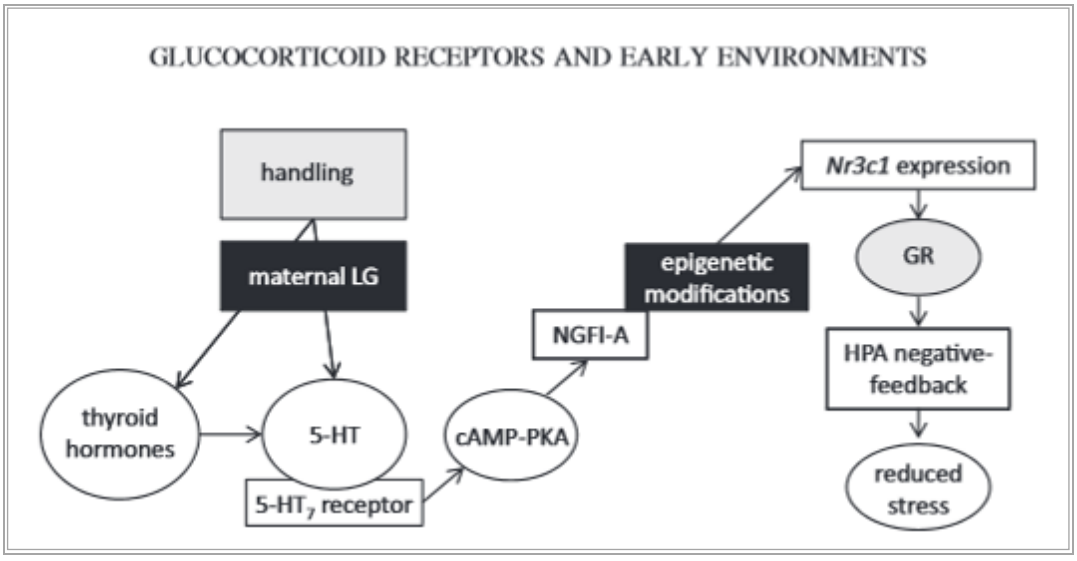

Figure 3. Molecular pathway between handling, maternal care, glucocorticoid receptors and stress response.

Handling has an influence on maternal LG, thyroid hormone levels and serotonin levels. Handling increases the serotonin levels which affect hippocampal GR concentration and leads to reduced stress response (Image source: Champagne 2013).

As already mentioned, maternal care plays a major role in influencing the hippocampal GR levels and affecting the control of the HPA axis in stressful situations (Liu et al. 1997). Adult offspring of high LG mothers showed increased GR expression in the

hippocampus compared to adult rodents reared by low LG dams (Francis et al. 1999). The range of GR expression is attributed to levels of methylation at the GR gene promoter where lower levels of methylation increase the access of transcription factor to promoter and chromatin regulatory proteins, like CREB binding protein, and therefore increase the histone acetylation at GR promoter, which is the case in high LG offspring (Martin et al. 2010).

In conclusion, epigenetic modifications are influenced by early life stress or MB and have long-lasting effects on the behavior and stress responsiveness of the individual which is often associated with neuropsychiatric disorders. The brain regions representing candidate sites as subjects of MB-induced epigenetic modifications which are also relevant to the neural circuitry involved in depression (Danielle L. Champagne et al. 2008; Bagot et al. 2012). Specifically, the role of the hippocampus in this context has been most intensively studied and will be introduced in the following section.

3.5 The Role of the Hippocampus in Depression

The hippocampus is a subcortical brain structure and part of the limbic system which is involved in learning and memory, but also in controlling emotions and has therefore been highly implicated in psychiatric disorders, including depression (Videbech & Ravnkilde 2004; Warner-Schmidt & Duman 2006; Bremner 1999; Wright 1997). Specifically, the ventral hippocampal region is responsible for the anxiety-related behaviors whereas the dorsal hippocampal area is more relevant for learning and memory (Fanselow & Dong 2010).

Particularly relevant to the pathomechanism of depression and response to antidepressant treatment is the subgranular zone of the dentate gyrus where adult hippocampal neurogenesis occurs (Dranovsky & Hen 2006; Kheirbek & Hen 2011; Mendez-David et al. 2013; Michael R. Drew & Hen 2007; Samuels & Hen 2011).
In MDD patients, neuroimaging studies showed that there is a functional deficit due to atrophy of hippocampal pyramidal neurons, decreased neuropil, altered morphology and reduction in hippocampal volume (Stockmeier et al. 2004; Sheline et al. 1996; Sheline et

al. 2003; Neumeister et al. 2005; Bremner et al. 2000; Kempton et al. 2011). However, hippocampal changes in depression and pertinent animal models are not restricted to the morphological and cellular levels, since also alterations in the molecular pathways, including levels of neurotransmitters, expression of neurotrophic factors and signaling molecules which lead to cellular destabilization and remodeling, have been described (Watanabe et al. 1992; Magariños et al. 1999; Radley et al. 2006; Radley & Morrison 2005; Radley et al. 2004, Duric & Duman 2013). These alterations have been also attributed to long-term stress exposure (Figure 4) with the resulting impairment being progressive and increasing with the duration of the subsequent disease (Duric & Duman 2013).

Figure 4

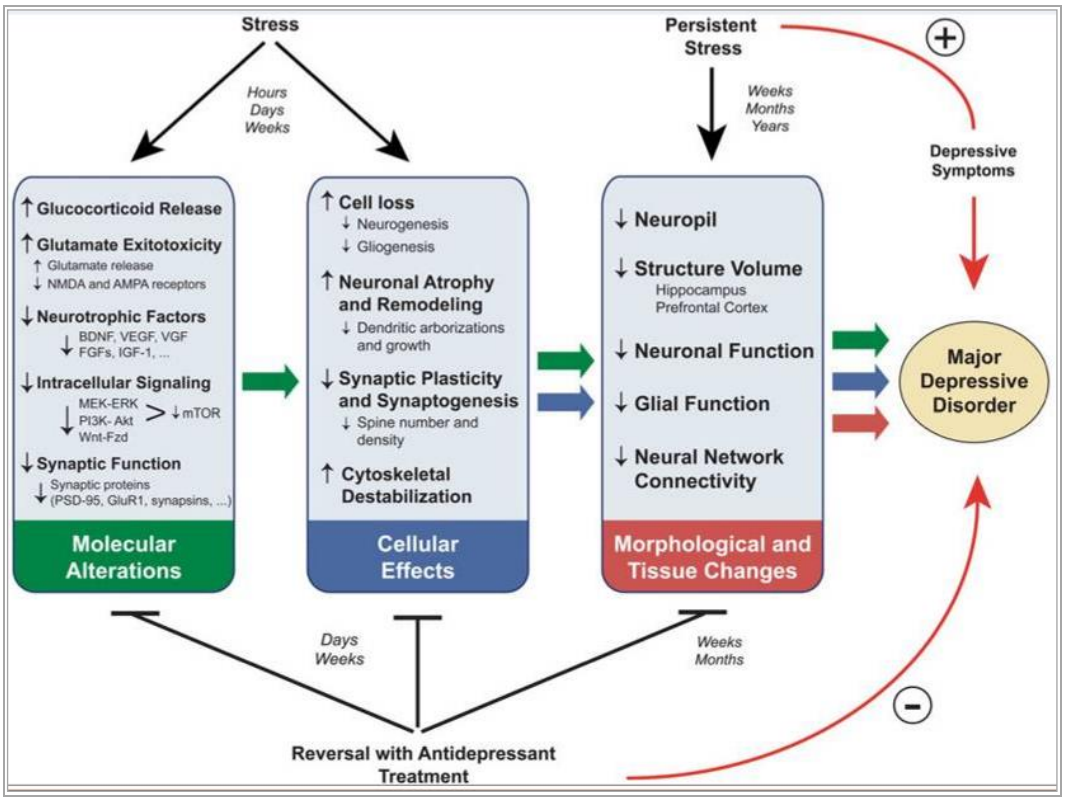

Figure 4. Effects of stress in molecular, cellular and structural pathways of MDD.

Stress induces molecular and cellular alterations, like changes in neurotrophic factor levels, increased glucocorticoid release, cell loss or cytoskeletal destabilization. Constant stress exposure can lead to morphological and tissue changes like decreased neuronal function and structure volume which results in MDD (Image source: Duric & Duman 2013).

4 Rationale

There is evidence for a modulatory impact of some forms of MIA on aspects of MB. However, a detailed characterization of MB after gestational treatment with Poly (I:C), known to induce augmented depression-like behavior in adult offspring, and the role of strain-dependent differences in MB of the mouse, has not been carried out so far.

5 Aims and Hypotheses

The aim of the present thesis was to employ two different inbred mouse strains to investigate whether genetically-determined distinctions in MB can modulate the disruptive impact of adverse early life events on emotional disturbances later in life, focusing on the effect of MIA on depression-like behavior.

Hence, we postulated and tested the following four hypotheses:

H1. Poly (I:C)-induced MIA at E12.5 exerts a disruptive effect on MB, specifically the frequency of licking/grooming.

H2. The effect of MIA on MB depends on the baseline level of pup-orientated behaviors, which differ between C57 and C3H mouse strains.

H3. MIA-induced alterations in MB are linked to depression-like behavior in adult offspring in a strain-dependent manner.

H4. Hippocampal expression of corticosteroid receptors constitutes a molecular interface between Poly (I:C)-induced MIA, MB and depression-like behavior later in life.

6 Experimental Design

Whether depression-like behavior resulting from MIA is modulated by maternal care and whether this effect is dependent on genetic susceptibility has not yet been investigated.

Therefore, pregnant dams were immunologically challenged at embryonic day 12.5 and the effect of MIA on maternal care, depression-like behavior and hippocampal corticosteroid receptor expression was evaluated in the F1 offspring in two different strains (C57, C3H).

7 Materials and Methods

7.1 Animals

C3H/HeNCrl (C3H) and C57BL/6N (C57) mice (Charles River, Germany) were used in the presented experiments.
Animals were single or group housed (corresponding to the experimental design) in standard polycarbonate cages with free access to food and water. The animal room was climate controlled under standard conditions with 12:12 light/dark-cycle (unless stated otherwise), 22°C and 50% humidity.
Animal experiments described in this study were approved by the national ethical committee on animal care and use (Bundesministerium für Wissenschaft und Forschung: BMWF-66.009/0015-II/3b/2012) and carried out according to international laws and policies.

The animals were group-housed during mating sessions and after birth until weaning at 4 weeks of age whereupon siblings were separated by sex and group-housed until 8 weeks of age when behavioral experiments were initiated. During the experimental period of behavioral analysis, animals were single housed.

7.2 Breeding

Male and female C3H and C57 were purchased from a certified vendor for timed-mating procedure (Meyer et al. 2005) described below which began after a 10 days acclimatization period. During this time females were group-housed (5 per cage) and male animals were kept single-housed in a separate animal room. Group housing of females was carried out in order to induce a synchronized anestrous phase (Lee-Boot effect) (Sahu 1982). In the subsequent co-housing phase, 4 female and 1 male mice were transferred into a partitioned cage without physical contact which allowed females to be exposed to male pheromones for 3 days (60-72h) in order to induce the

synchronized estrous cycle (receptive phase) due to the Whitten effect (Ochiogu et al. 2010). Additionally, female bedding was replaced by soiled male bedding in the partitioned cage.

In the following mating phase, one female was moved to a male cage overnight (6pm to 8am). The next morning, the male was removed from the cage and the presence of vaginal plugs and female body weights were recorded. This time point was denoted embryonic day 0.5 (E 0.5). Two paper towels per cage were provided to encourage nest-building and females were left undisturbed except for documentation of body weights on embryonic day 6.5 and 12.5. Females with over 16% weight gain (Hau & Skovgaard Jensen 1987) were assumed to be pregnant and treated as control (saline vehicle) or experimental (Poly (I:C) mice (details below). Upon delivery (P0) the collective weight of each litter and number of pups were recorded.

7.3 Maternal Immune Activation (MIA)

Maternal immune activation paradigm followed a previously established procedure (Khan et al. 2014). Briefly, Polyinosinic:Polycytidylic acid (Poly (I:C)) phosphate salt (Sigma-Aldrich Cat# P9582) was dissolved in 0.9% physiological saline and administrated intraperitoneal (i.p., 20 mg/kg) to pregnant dams on E12.5. Control groups received saline only. The volume of all injections was held constant at 10ml/kg.

7.4 Maternal Behavior (MB)

MB was evaluated according to a standardized protocol (Franks et al. 2011). Briefly, MB was recorded by videotaping twice a day for six consecutive days starting at postnatal day 1 (P1). To this end, video recording using commercially available webcams (Logitech C525 HD Webcam, Microsoft LifeCam HD-3000, Trust Widescreen HD-Webcam, Creative LIVE! Cam Chat HD USB-Webcam) was initiated and ended manually by a remote-system (© TeamViewer 2014 GmbH) over the Internet with the first daily recording between 11am and 1pm and the second from 3pm to 5pm. Webcams were placed in front of the home cages with the mother and their pups in it. At

the end of the six day observation period, videos were analyzed by a trained experimenter blind to the experimental condition by the documentation of MB every 3min (total of 40 observations per session, 80 per day) as previously described (Franks et al.). Behavioral events recorded were categorized as pup-oriented parameters (licking/grooming of pups and nursing) and non-pup-oriented parameters (nest-building, self-grooming, eating, drinking, sleeping, extras (like climbing in the cage, digging, etc.)). The analysis consisted of calculating the percentage of time a mother engaged in each of the behavioral parameters in correspondence to the total observation time for each day.

7.5 Behavioral Testing

Mice were single-housed one week before starting behavioral testing. All behavioral experiments were carried out during the light phase of the animals' light/dark-cycle and mice were allowed to habituate to the experimental room for 20min every day prior to start of experiments.

7.5.1 Sucrose Preference Test (SPT)

The sucrose preference test assessing anhedonic behavior was carried out according to a standard procedure (Pollak et al. 2008; Yu et al. 2007; Monje et al. 2011).
To prepare the mice for the SPT, they had to be trained with sucrose-solution for 2 days. 18h prior to the test the animals were deprived of food and water. During the 3h testing period the mice got the chance to choose between two identical bottles which were placed in the home cage. One bottle contained 2% sucrose solution (Sucrose, Sigma Aldrich) and the other one tap water. The weight change of the bottles before and after the test was evaluated in order to calculate the volume of the liquid consumption.

7.5.2 Open Field (OF)

The OF procedure was adapted from a standard protocol (Khan 2014).

Locomotor activity in the open field was recorded for 60min by computational tracking system (Activity monitor, Med Associates inc., SOF 811). Before starting the experiment the level of illumination was measured in each of the 4 chambers and the light levels were adjusted to 180-300 lux.

The mice were placed in the center of the chambers (size: 100cm diameter, 32cm height) at the on-set of the experiment. By setting the parameters of the program according to the user's manual (Activity monitor user's manual, Med Associates inc, 2009) the chambers were divided into 2 zones, where zone 0 is the outside location and zone 1 is the center location. The total distance travelled and the entries into the center and the periphery zone of the chambers were automatically monitored by the software (Activity monitor, Med Associates inc., SOF 811).

7.5.3 Light Dark Box (LD)

LD experiments were carried out using a standard procedure (Khan 2014) and conducted by placing a designated insert into the OF test chambers (Activity monitor, Med Associates inc.).

At the beginning of the 10min testing time, animals were placed into the dark compartment and allowed to move freely between the two sides of the chamber. The locomotor activity and the amount of time the animals spent in the light, dark or transition area and the zone entries were automatically recorded by the software (Activity monitor, Med Associates inc., SOF 811).

7.5.4 Elevated Plus Maze (EPM)

The EPM widely used as test for innate anxiety in rodents consisted of a plus maze, constructed with two open and two closed arms (each size: 45x10cm, height: 50cm) and elevated approximately 65cm above the floor. The light levels were carefully measured and configured to 30 lux in closed and 100 lux in open arms. Animals were tracked for

5min by an automated tracking system (VIDEOTRACK [03-PLUSMAZE-NSHD-LBW], Viewpoint, France)

Each mouse was placed in the center (5cm x 5cm) of the maze facing an open arm at the beginning of the observation period. Parameters which were monitored by the software included total distance travelled, time spent in open or closed arms and number of entries (Khan 2014).

7.5.5 Rota Rod (RR)

The rota rod and the computational monitoring system (USB Rota Rod “SOF-ENV-57X”, MedAssociates Inc., St. Albans USA) were used to evaluate motor coordination of rodents according to a previous used protocol (Khan 2014; Rogers et al. 1999). The set-up contains a rotating rod which was accelerated from 4-40 RPM. The motor balance of the animal was examined by the latency it could stay on the rotating drum while accelerated. The experiment lasted for a maximum of 5min and stopped automatically when the horizontal infrared beams were broken through the fall of a mouse from the rod. The time till the animal fall from the rod and the speed of rotation was recorded by the software. Each mouse had the chance to habituate for 30 seconds on the rod without acceleration. The experiment was repeated for 3 times and the average of the time of an animal balancing on the rod was evaluated.

7.5.6 Forced Swim Test (FST)

The FST, which measures depression-related behavioral despair, was carried out according to a standardized protocol (Pollak et al. 2008; Dulawa et al. 2004; Khan 2014). The mice were placed in a Plexiglas beaker (size: 25cm diameter, 46cm deep) filled with 21-23°C water to a height of 36cm and the tracking software (VIDEOTRACK [PORSOLT] software, Viewpoint, France) started automatically. The behavior of the mice was monitored with an infrared video camera. Immobility (only movements to prevent drowning) was scored for the last 4min of the total test period (6min). The water was replaced by fresh water between each test. Afterwards animals were returned to their home cages and dried with towels and a red-light lamp.

7.6 Dissection

Animals were sacrificed by neck dislocation and the brain was rapidly dissected out and the hippocampi were separated from the rest of the brain tissue. Samples for subsequent mRNA analysis were stored in RNase-free Eppendorf tubes containing RNAlater® (Ambion, Austin, TX, USA).

7.7 RNA Extraction

RNA was carried out using Qiagen miRNA Mini Kit according to the manufacturer's instructions (Qiagen® miRNeasy Mini Handbook).

Briefly, tissue was removed from RNAlater and excess of liquid was dried using a sterile filter paper before submerging the sample into 700µl QIAzol (Phenol/guanidine-based QIAzol® Lysis Reagent) and homogenization using a mini-hand mixer. After 5min incubation period the samples were centrifuged at 10000rpm for 2min. Subsequently, 140µl Chloroform (CH3Cl) was added, samples were vortexed and centrifuged again at 12000rpm (4°C) for 15min. The upper aqueous phase (350µl) was transferred into a new collection tube and 1.5 volumes (525 µl) of 100% ethanol (EtOH) were added. 700µl of the solution was pipetted into a RNeasy Mini column and centrifuged at 10000rpm (RT) for 15sec. The flow-through was thrown away and the step was repeated with the rest of the sample. Next, 700µl of RWT-buffer was added into the RNeasy Mini column and centrifuged at 10000rpm (RT) for 15sec. The flow-through was discarded again and 500µl of RPE-buffer was added into the column and centrifuged at 10000rpm (RT) for 15sec. The last step was repeated one more time but with a final centrifugation step of 2min. The flow-through was discarded and the RNeasy Mini column was placed into a new 2ml collection tube and centrifuged at 16000rpm (RT) for 1min. The RNeasy Mini column was transferred into a new 1.5ml RNase-free Eppendorf tube and 40µl of RNase-free water was added into the column and centrifuged at 10000rpm (RT) for 1min. The eluent was pipetted again onto the column and the same centrifugation step was repeated. Finally, RNA concentration and purity were determined using a Nanodrop photometer (NanoPhotometer^{TM}, IMPLEN, 7122 V2.3.1) and samples were stored at -80°C until used for further analysis.

7.8 cDNA Synthesis

The cDNA synthesis was performed using the cDNA synthesis DyNamo Kit (Thermo Scientific) following the provided instructions with all steps being carried out on ice. At first, the samples were thawed and the required volume (containing 900ng of total RNA) for each sample was transferred into a new tube and RNase-free water was added up to a final volume of 7µl. The master mix which contained Reverse Transcriptase buffer, Random Hexamer Primer set and M MulV RNase H+ reverse transcriptase (MMLV Reverse Transcriptase 1st-strand cDNA synthesis Kit, epicenter, cat.no. MM070150, USA) was prepared following the provided instructions and 13µl were added to each sample. Samples were placed into a thermocycler and incubated for 45min in a step-wise temperature protocol (primer extension: 10min at 25°C; cDNA synthesis: 30min at 37°C; reaction termination: 5min at 85°C; cooling of the sample: at 4°C). Samples are stored in a freezer at -20°C.

7.9 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The Qiagen miScript SYBR® Green PCR Kit (cat.no. 2951948) was used for qRT-PCR analysis. The primers were adapted from Lin et al. 2011 and used at a final concentration of 0.2µM. cDNA samples were thawed on ice and 4,2µl of each sample were added to a new tube containing 26,04µl RNase free water. Primer-specific mastermixes were prepared and 7.8µl mastermix and 7.2µl sample (or RNase free water for blank) were pipetted into a 96-well plate. The PCR plate was sealed with foil and centrifuged at 1000rpm for 5min. Then the plate was placed into the thermocycler (Applied Biosystems®, life technologies™) and the software (StepOne Software v2.2.2 Applied Biosystems®, life technologies™) was programmed according to the PCR-Protocol (initial denaturing: 10min at 95°C; continued by max. 40 cycles; denaturing: 15sec at 95°C; annealing temperature: 30sec at 60°C; elongation temperature: 30sec at 60°C).

7.10 Statistical Analysis

All data were analyzed with using Microsoft Excel 2010 or IBM SPSS Statistics 21 software packages. 2-way and 3-way ANOVA were employed for the analysis of maternal behavior and results of qRT-PCR in a 2 x 2 or 3 x 2 set-up: treatment (Poly (I:C) vs. Saline) x strain (C57 vs. C3H) x sex (female vs. male). For the evaluation of behavioral testing in adult offspring Student's t-tests were carried out between control and MIA groups. The level of significance was defined as $p<0.05$ in all instances.

8 Results

8.1 Maternal Behavior is Disrupted upon Poly (I:C)-Induced MIA at E12.5 in C57 and C3H Mice.

In order to evaluate the effects of MIA on maternal care of C3H and C57 mice, different parameters of maternal behavior, categorized as pup-orientated (LG and nursing) and non-pup-orientated (nest-building, self-grooming, eating, drinking, extras and sleeping) were analyzed from postnatal day 1 to 6 according to an established protocol (Franks et al. 2011).

8.1.1 C57 and C3H Mothers Display Differential Levels of Pup-Orientated and Non-Pup-Oriented Behavior.

A significant main effect of strain ($F_{(2,44)}$=26.997; $p<0.001$) was observed for pup-oriented behavior with C3H engaging in a higher percentage of time in pup-oriented behavior than C57 mice. A corresponding effect on non-pup-oriented behavior was revealed (main effect of strain ($F_{(2,44)}$=23.971; $p<0.001$)) where C57 had more non-pup-oriented behavior compared to C3H mice. But in addition no effect of treatment ($F_{(2,44)}$=0.918; p=0.344; $F_{(2,44)}$=1.360; $p>0.05$) or interaction between treatment and strain ($F_{(2,44)}$=0.265; $p>0.05$; $F_{(2,44)}$=0.083; $p>0.05$) for pup-oriented and non-pup-oriented behavior was detected (Figure 5 and 6).

Figure 5

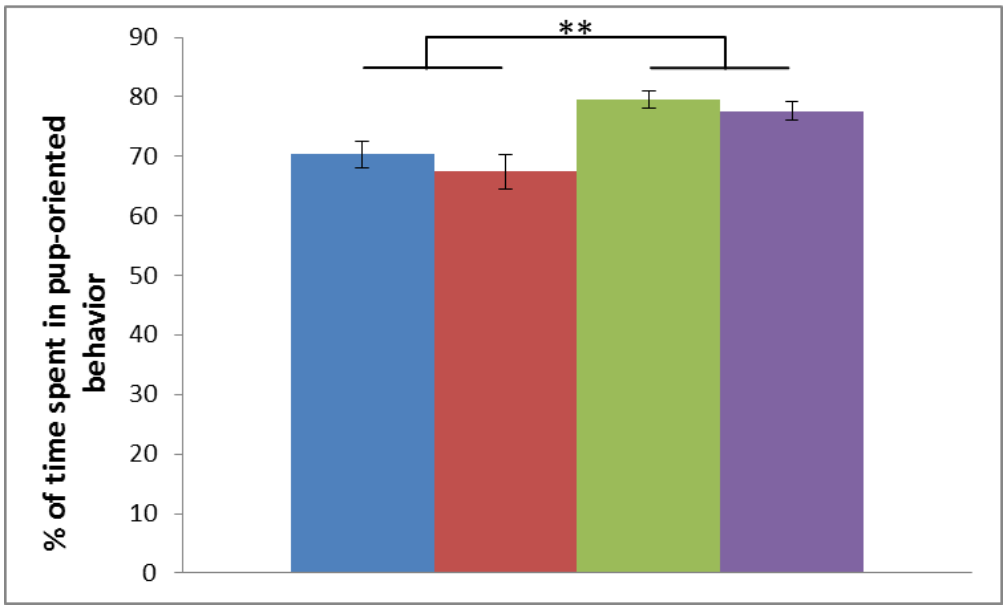

Figure 5. Pup-oriented behavior of C57 and C3H dams.
Percentage of total time C57 and C3H mothers of control (blue and green) and Poly (I:C) (red and purple) groups spent engaging in pup-orientated behavior (n=8-14 per group). Data are presented as mean ± SEM. **p<0.01.

Figure 6

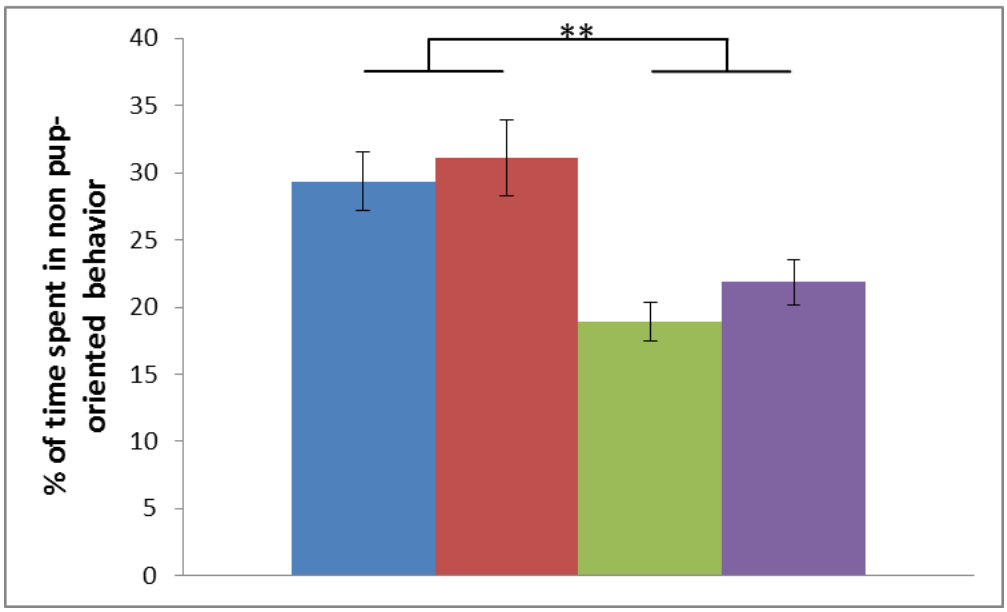

Figure 6. Non pup-oriented behavior of C57 and C3H dams.
Percentage of total time C57 and C3H mothers of control (blue and green) and Poly (I:C) (red and purple) groups spent engaging in non-pup-orientated behavior (n=8-14 per group). Data are presented as mean ± SEM. **p<0.01.

8.1.2 Poly (I:C) Treatment Reduces the Frequency of Licking/Grooming in C57 and C3H Mothers but Does not Affect Other Aspects of Pup-Orientated Maternal Behavior.

A significant main effect of treatment ($F_{(2,44)}$=24.525; p<0.001) on LG behavior was revealed with Poly (I:C) treated animals displaying significantly less LG behavior towards their offspring from P1 to P6. However, no effect of strain ($F_{(2,44)}$=1.561; p>0.05) or interaction between treatment and strain ($F_{(2,44)}$=1.461; p>0.05) on LG behavior was exhibited (Figure 7).

Figure 7

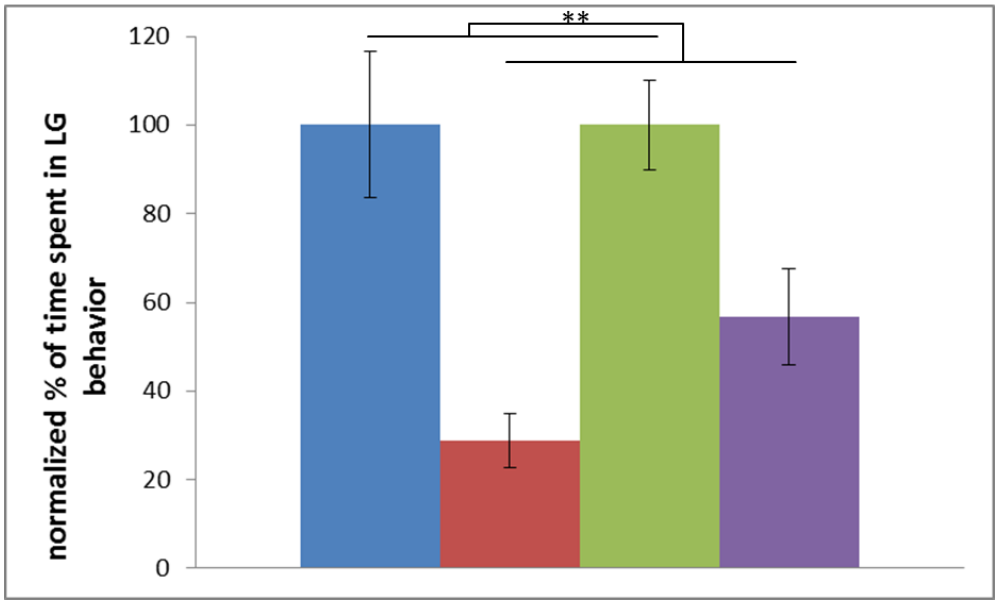

Figure 7. LG of C57 and C3H dams.
Normalized percentage of total time C57 and C3H mothers of control (blue and green) and Poly (I:C) (red and purple) groups spent engaging in LG behavior (n=8-14 per group). Data are presented as mean ± SEM. **p<0.01.

No significant main effect or interactions for the other parameters representing pup-orientated behavior (i.e. nursing) were detected (Table 1).

8.1.3 Effects of MIA on Specific Parameters of Non-Pup-Orientated Maternal Behavior.

2-way ANOVA revealed a significant main effect of treatment ($F_{(2,45)}$=7.792; p<0.05) on nest-building with higher levels observed in Poly (I:C) while no effects of strain ($F_{(2,45)}$=0.006; p>0.05) or interaction between treatment and strain ($F_{(2,45)}$=0.006; p>0.05) were observed (Figure 8).

Figure 8

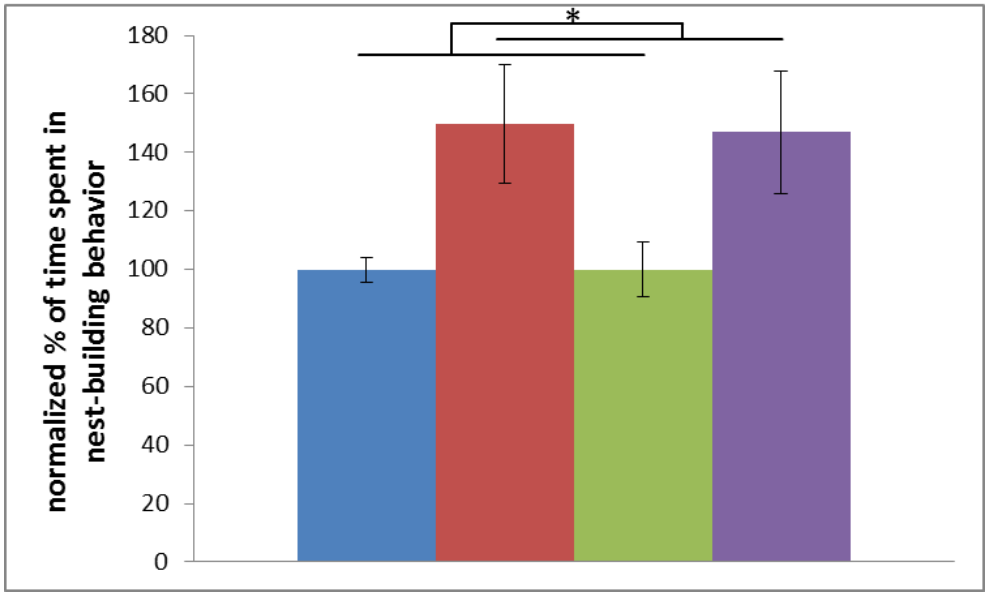

Figure 8. Nest-building of C57 and C3H dams.
Normalized percentage of total time C57 and C3H mothers of control (blue and green) and Poly (I:C) (red and purple) groups spent engaging in LG behavior (n=8-14 per group). Data are presented as mean ± SEM. *p<0.05

Additionally, significant main effects of treatment ($F_{(2,44)}$=5.696; p<0.05) for self-grooming and drinking ($F_{(2,44)}$=10.810; p<0.05) were observed (Table 1). While for self-grooming no additional main effects of strain or interaction were detected. A significant main effect of treatment, strain ($F_{(2,44)}$=11.577; p<0.05) and interaction between strain and treatment ($F_{(2,44)}$=11.577; p<0.05) was found for drinking behavior (Table 1).
Lastly, for the parameter “extras” which comprises activities like running and climbing in the cage a significant main effect of treatment ($F_{(2,46)}$=4.808; p<0.05), without an effect of strain or interaction between treatment and strain was observed (Table 1).

Table 1 a.)

	C57		C3H	
Parameters	**Control**	**Poly (I:C)**	**Control**	**Poly (I:C)**
nursing	100 ± 1.86	101.08 ± 4.55	100 ± 1.86	96.39 ± 1.85
self-grooming	100 ± 19.50	149.71 ± 22.72	100 ± 14.5	131.67 ± 18.49
eating	100 ± 9.85	90.27 ± 10.37	100 ± 7.11	125.32 ± 13.45
drinking	100 ± 28.23	375.44 ± 79.93	100 ± 15.07	95.28 ± 16.68
extras	100 ± 4.96	120.65 ± 11.31	100 ± 14.54	146.30 ± 17.40
sleeping	100 ± 19.56	80.36 ± 40.84	100 ± 34.86	131.33 ± 46.53

Table 1 b.)

	Strain		Treatment		strain * treatment	
Parameters	**F (df)**	**p**	**F (df)**	**p**	**F (df)**	**P**
nursing	0.748 (2,45)	0.392	0.218 (2,45)	0.643	0.746 (2,45)	0.393
self-grooming	0.280 (2,44)	0.600	5.696 (2,44)	0.022	0.280 (2,44)	0.600
eating	2.450 (2,46)	0.125	0.485 (2,46)	0.490	2.450 (2,46)	0.125
drinking	11.577 (2,44)	0.002	10.810 (2,44)	0.002	11.577 (2,44)	0.002
extras	0.706 (2,46)	0.405	4.808 (2,46)	0.034	0.706 (2,46)	0.405
sleeping	0.386 (2,44)	0.538	0.020 (2,44)	0.887	0.386 (2,44)	0.538

Table 1. Maternal behavior parameters of C57 and C3H dams.

a.) Summary of the goup-wise results (mean ± SEM) of selected paramteres of maternal behavior in C57 and C3H control and Poly (I:C) animals (normalized to C57 controls; n=8-14 dams per group). **b.)** Results of the statistical evaluation using 2-way ANOVA for the detection of main effects (strain, treatment) and interaction term (strain*treatment).

8.2 Poly (I:C)-Induced MIA Does not Modulate Anxiety and Depression-Like Behavior in Adult C3H Offspring.

In order to examine whether, as previously described for C57 offspring, Poly (I:C)-dependent MIA also modulate depression-like behavior in C3H adult male and female offspring, two well-established tests to independently examine different aspects of rodent behavior related to depression in humans, were employed: the Sucrose Preference Test (SPT), a routinely used paradigm for the evaluation of anhedonic behavior (Willner et al. 1987) and the Forced Swim Test (FST), a standard test for the assessment of depression-related behavioral despair (Can et al. 2012).

In order to test potential effects of prenatal Poly (I:C) treatment on anxiety-like behavior, the Elevated Plus Maze (EPM) and the Light Dark box (LD) were used. Additionally, in order to reveal possible unspecific effects of MIA on other behavioral aspects which could confound the performance in depression- and anxiety-related tests, locomotor activity was evaluated in the Open Field (OF) and motor-coordination in the Rota Rod (RR).

No significant main effect of treatment or interaction between sex and treatment for depression-related behavioral phenotypes was detected. However, In the FST a significant main effect of sex ($F_{(2,44)}$=5.488; $p<0.05$) in "% of immobility" was revealed since males of both treatment groups spent higher % of time in immobility than females (Table 2).

In the LD, a significant main effect of sex ($F_{(2,68)}$=4.273; $p<0.05$) on "total ambulatory distance" was found, with females of both treatment groups travelling larger distances than males (Table 2). In the EPM, a significant effect of the interaction between sex and treatment ($F_{(2,56)}$=5.287; $p<0.05$) was detected in "time spent in open arms" but no main effect of sex or treatment was revealed (Table 2).

For the OF the only significant effect detected by the 2-way ANOVA was a main effect of treatment ($F_{(2,66)}$=7.014; $p<0.05$) on "center zone entries" with Poly (I:C) injected animals showing more entries into the center than control mice (Table 2).

No differences induced by either treatment or sex or an interaction between the two factors were observed in the other parameters of OF (Table 2).

Table 2 a.)

		Control		Poly (I:C)	
Test	**Parameters**	**Female**	**Male**	**Female**	**Male**
SPT	sucrose consumption [%]	87.92 ± 1.16	83.93 ± 2.91	85.31 ± 2.53	81.11 ± 2.03
OF	Total ambulatory distance [cm]	5481.07 ± 301.79	5209.17 ± 350.57	6020.71 ± 548.46	5647.03 ± 276.54
	Time in center [%]	3.80 ± 0.33	4.10 ± 0.42	4.66 ± 0.49	5.07 ± 0.38
	Center zone entries	106.44 ± 10.20	106.65 ± 8.57	143.18 ± 15.63	124.94 ± 7.25
LD	Total ambulatory distance [cm]	1810.77 ± 117.55	1540.74 ± 77.77	1803.98 ± 154.49	1583.16 ± 119.45
	Time in light zone [%]	40.18 ± 2.41	40.66 ± 2.76	37.92 ± 3.16	32.51 ± 4.09
	Light zone entries	27.67 ± 2.61	29.85 ± 3.51	32.27 ± 3.43	21.28 ± 3.17
EPM	Time in open arms [s]	39.87 ± 3.65	53.27 ± 8.60	43.94 ± 8.42	25.27 ± 4.93
RR	Latency to fall [s]	89.98 ± 6.28	101.02 ± 4.22	92.46 ± 10.25	85.80 ± 5.33
FST	Immobility [%]	15.98 ± 2.11	18.23 ± 2.28	12.43 ± 2.32	22.60 ± 2.85

Table 2 b.)

		Sex		treatment		sex * treatment	
Test	**Parameters**	**F (df)**	**p**	**F (df)**	**p**	**F (df)**	**p**
SPT	% of sucrose consumption	2.909 (2,60)	0.094	1.277 (2,60)	0.263	0.002 (2,60)	0.963
OF	Total ambulatory distance [cm]	0.738 (2,65)	0.394	1.692 (2,65)	0.198	0.018 (2,65)	0.893
	Time in center [%]	1.973 (2,65)	0.165	2.398 (2,65)	0.127	0.202 (2,65)	0.655
	Center zone entries	0.753 (2,66)	0.389	7.014 (2,66)	0.010	0.788 (2,66)	0.378
LD	Total ambulatory distance [cm]	4.273 (2,68)	0.043	0.023 (2,68)	0.881	0.043 (2,68)	0.836
	Time in light compartment [%]	0.521 (2,66)	0.473	2.326 (2,66)	0.132	0.743 (2,66)	0.392
	Light zone entries	1.630 (2,67)	0.206	0.330 (2,67)	0.568	3.645 (2,67)	0.061
EPM	Time open arms [s]	0.116 (2,56)	0.735	2.385 (2,56)	0.129	4.287 (2,56)	0.043
RR	Latency to fall [s]	0.121 (2,68)	0.730	1.023 (2,68)	0.316	1.970 (2,68)	0.165
FST	immobility [%]	5.488 (2,64)	0.022	0.024 (2,64)	0.878	2.230 (2,64)	0.141

Table 2. Behavioral evaluation in adult male and female C3H mice offsprings after prenatal Poly (I:C) treatment and controls.

Depression- and anxiety-like behavior was analysed using SPT, OF, LD, EPM, RR and FST.

a.) Summary of the goup-wise results (mean ± SEM) behavioral analysis in male and female adult offspring after Poly (I:C)-induced MIA and control mice (n=11-21 animals per group). b.) Results of the statistical evaluation using 2-way ANOVA for the detection of main effects (sex and treatment) and interaction term (sex*treatment).

8.3 Hippocampal Corticosteroid Receptor Expression is Modulated by Sex, Strain and MIA-Treatment in Adult Offspring of C57 and C3H Mice.

Aiming to unravel potential molecular correlates of the observed differential behavioral responses of C57 and C3H offspring to prenatal Poly (I:C) exposure, we investigated GR and MR expression in Poly (I:C) and control adult offspring. To this end, qRT-PCR analysis using hippocampal tissue of male and female C57 and C3H offspring was carried out. 3-way ANOVA was used to determine main effects of strain (C57 and C3H), sex (females and males) and treatment (saline and Poly (I:C)) and interaction terms for GR and MR respectively.

Significant main effects of interaction sex*treatment ($F_{(3,61)}$=4.665; $p<0.05$) and strain*treatment ($F_{(3,61)}$=4.382; $p<0.05$) were found in GR levels (Figure 9). Additionally, for MR expression, a significant main effect of the interaction of strain*treatment ($F_{(3,61)}$=14.806; $p<0.05$) was detected (Figure 10) without further significant main effects or interactions.

Figure 9

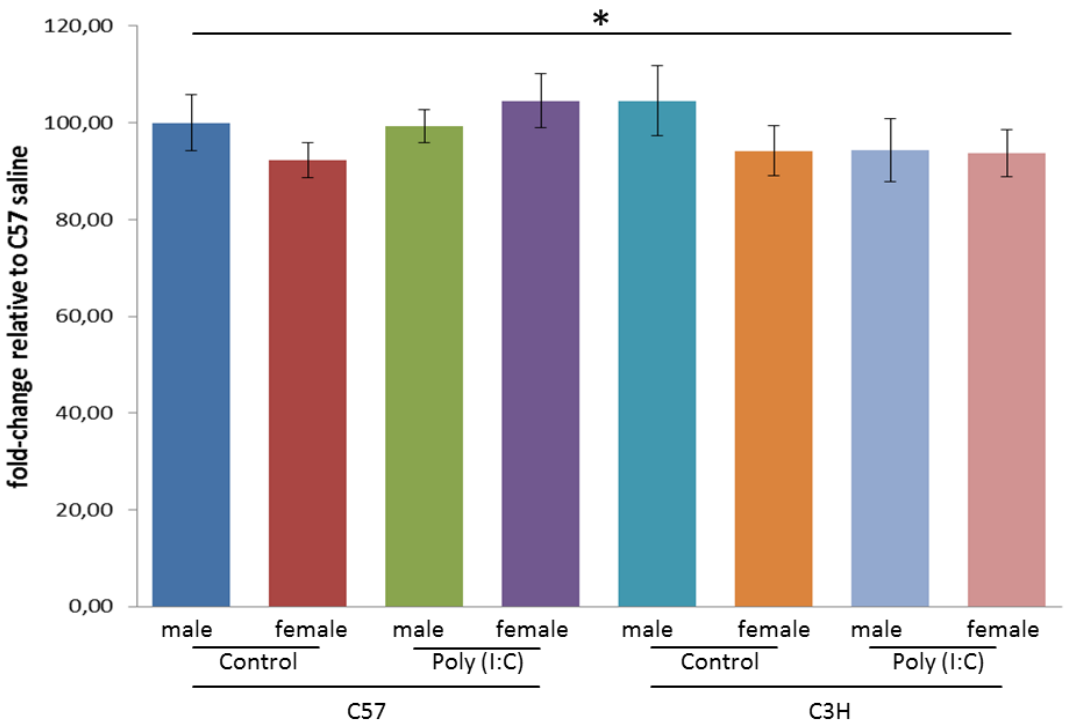

Figure 9. Hippocampal GR expression in adult male and female C57 and C3H mice after prenatal Poly (I:C) treatment and controls.

Expression levels were determined by qRT-PCR and relative values (ΔCT) were normalized to the C57 male saline average. Significant effect of interaction strain*treatment and sex*treatment is shown. Data are presented as mean± SEM (n=8 per group). *p<0.05

Figure 10

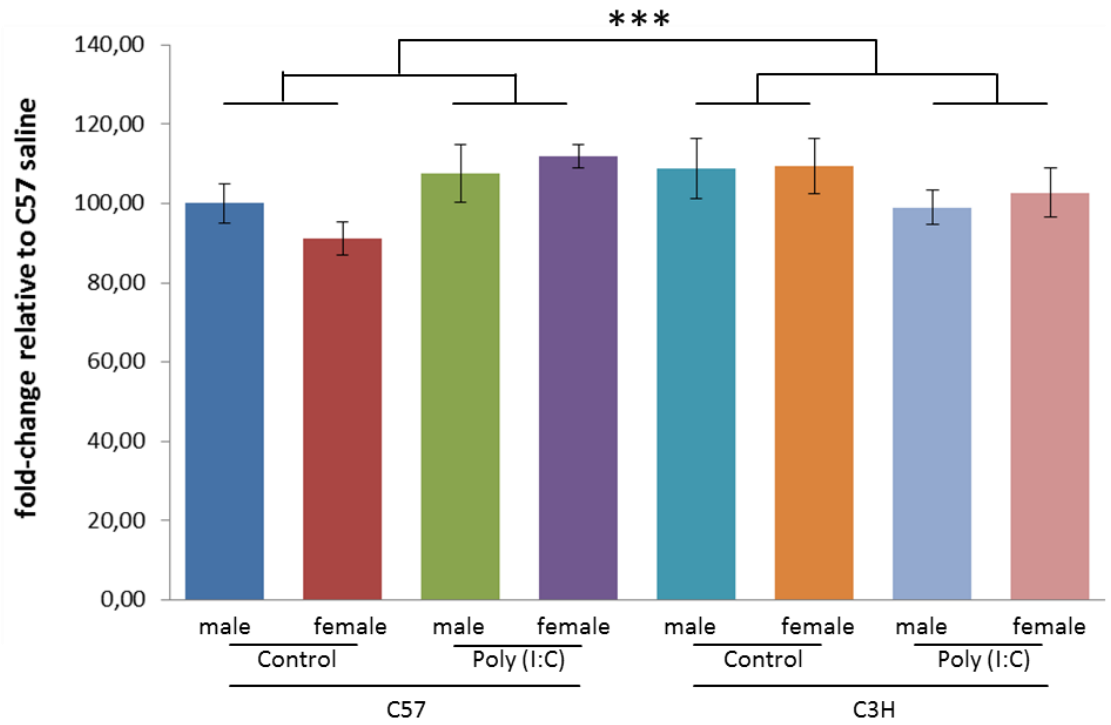

Figure 10. Hippocampal MR expression in adult male and female C57 and C3H mice after prenatal Poly (I:C) treatment and controls.

Expression levels were determined by qRT-PCR and relative values (ΔCT) were normalized to the C57 male saline average. Data are presented as mean± SEM (n=8 per group). ***p<0.001

9 Discussion

The rationale for the present study - summarized in this master thesis - was to investigate the impact of MIA on maternal behavior and its association to depression-like behavior in adult offspring, specifically focusing on the relevance of the genetic background, and to examine potential molecular correlates.

9.1 Maternal Behavior is Disrupted upon Poly (I:C)-Induced MIA at E12.5 in C57 and C3H Mice.

The first two hypotheses of this study are that Poly (I:C) induced MIA has an disturbing effect on MB, and that this effect is dependent on genetically determined baseline levels of pup-oriented behaviors. Different parameters of MB (pup-oriented behavior: LG, nursing; non-pup-oriented behavior: nest-building, self-grooming, eating, drinking, sleeping and extras) were observed in dams of two different inbred mouse strains (C3H and C57) after immune stimulation treatments with Poly (I:C) at E12.5 and in saline treated controls.
C3H and C57 mouse strains have been selected based upon published evidence for differential pup-oriented behavior (Koehl et al. 2012; van der Veen et al. 2008), which was confirmed in the present study with C3H dams presenting higher levels of pup-oriented behavior. One possible cause for this strain-dependent characteristics with regard to maternal behavior could be anatomical differences in the medial preoptic nucleus (MPOA), a major region in the hypothalamus regulating MB (Numan 2007) as it has been previously described in other mouse strains (Mathieson et al. 2002).

In terms of the neural circuitry, strain-dependent differences in maternal behavior could be related due to the interstrain variability in dopaminergic signaling (Ng et al. 1994; Colelli et al. 2010) which is known to be critical for motivated behaviors, including maternal behavior (Henschen et al. 2013). Indeed, C3H mice have been reported to have approximately 50% higher levels of dopamine 2- receptors (D2R) than mice of the C57 strain (Boehme & Ciaranello 1981; Helmeste & Seeman 1982). Hence D2R, which

has been shown to be critical for MB (Byrnes et al. 2002) is also a highly likely molecular correlate of differential pup-orientated behavior among these two inbred mouse strains. Additionally, molecular elements of the hormonal systems, known to induce MB through their effects on the MPOA (including estrogen, prolactin and oxytocin) (Bridges et al. 1990; Numan et al. 1977; Pedersen et al. 1994), and natural variations determined by the specific genetic background may both contribute to the observed relative reduction of pup-orientated behaviors in C57 as opposed to C3H mothers. However, strain-specific expressional patterns of these hormones and their respective receptors in relation to maternal performance remain to be investigated in future studies. Considering the dampening effects of Poly (I:C)-induced MIA on MB which override the genetically determined differences, the present results are in agreement with previous reports in academic literature, describing disruption of MB upon prenatal LPS exposure (Soto et al. 2013; Penteado et al. 2014). There are other studies though, which did not find an effect of gestational LPS treatment on MB (Baharnoori et al. 2012). Differences in the experimental design, including the exact timing of MIA and the dosage applied, may account for the observed different outcomes. However, aberrant MB has not only been reported as a result of infectious stress but is also known to result from other forms of gestational stress (Baker et al. 2008). Hence, key players of the endogenous stress response system are likely involved in transmitting the effects of both psychosocial and infectious stress on maternal behavior. Indeed, differences in GR expression, linked to variations in maternal care (Chourbaji et al. 2011), and the prominent role of the GR as molecular interface, mediating gene x environment interactions, jointly contributing to long-term changes in brain function as well as associated behavioral outputs (Daskalakis et al. 2013), are inviting future experimental investigations aiming to examining the specific role of GR in the neural events leading to the repressive effects of MIA on MB.

9.2 Poly (I:C)-Induced MIA Does not Modulate Anxiety and Depression-Like Behavior in Adult C3H Offspring.

The third hypothesis of the present study is that the effects of MIA on depression-like behavior later in life depend on the genetic background, hence leading to strain-dependent behavioral manifestations.

Specifically, we aimed to define whether genetically determined differences in MB may interact with the effects of MIA on depression-like behavior in adulthood by comparing the performance of adult C3H offspring after Poly(I:C)-induced MIA and controls to previously reported observations in the C57 strain. Although depression is a human psychopathology, some of the symptoms experienced by depressed patients, including anxiety, feeling of helplessness and the inability to experience pleasure (anhedonia), can also be evaluated by specific behavioral tests in animal models. Here, the effect of MIA on depression-like behavior was examined using specific behavioral assays, including the SPT and the FST, internationally accredited and widely used behavioral paradigms in preclinical depression research (Salazar et al. 2012; Pollak et al. 2010; Pollak et al. 2008). Furthermore, possible alterations of other behavioral features, including anxiety-like behavior, exploratory and locomotor activity as well as motor coordination, were determined in adult MIA and control C3H male and female offspring using routinely employed, well-validated standard assays. Therefore any additional effects of MIA potentially confounding results in the depression tests can be excluded.

However, not only the genetic background plays a role in influencing depression-like behavior. The test situation itself is dependent on many external variables, including physical environmental factors (e.g. light or noise), or manipulation before testing (e.g. housing or handling) (Bogdanova et al. 2013; Pothion et al. 2004; Mineur et al. 2006).

The evaluation of depression-like behavior in the FST, assessing behavioral despair, which is supposed to mimic the feeling of helplessness commonly reported by depressed patients (Abelaira et al. 2013), revealed sex-dependent performance in C3H mice, previously also reported in C57 mice (Khan 2014).

In agreement with our results, previous reports in scientific literature support the observation that females display less immobility than males in the FST (Alonso et al.

1991; Barros & Ferigolo 1998; Brotto et al. 2000), which is thought to be independent of ovarian hormones since the results of females is similar in different phases of the estrous cycle (Alonso et al. 1991). Nevertheless, Barros & Ferigolo 1998 showed that the effects of the antidepressant imipramine in the FST is different between males and females according to the different stages of estrus and proestrous.

Furthermore, male offspring of early prenatal stress show maladaptive behaviors in the FST and the tail suspension test (TST), with higher immobility than control mice, due to stress-induced learned helplessness, while by contrast, no effect was observed in female offspring in these tests (Mueller & Bale 2008). Interestingly, we observed that MIA did not affect the "% of immobility", which should display the main parameter reflecting depression-related behavioral despair, in either male or female CH3 offspring, contrary to the report of previous results obtained in male C57 mice (Khan et al. 2014). Similarly, no effect of MIA on sucrose preference in the SPT, reflecting depression-associated anhedonic behavior in mice (Salazar et al. 2012), was detectable in the C3H strain, while in the C57 strain significantly reduced sucrose preference has been previously reported in offspring after Poly (I:C)-induced MIA (Khan et al. 2014). While the description of the impact of MIA on depression-like behavior in C57 also includes a significant main effect of sex, this finding was not paralleled by respective sex-dependent performance of C3H mice in the SPT.

Considering the high degree of comorbidity between depression and anxiety disorders (Hirschfeld 2001), the next step in the characterization of MIA-effects on adult offspring phenotype consisted of evaluating anxiety-like behavior. To this end, we used two of the most commonly used behavioral assays, namely the EPM and the LD test. Both tests are based on experiencing a conflict situation. The animal has to resolve the situation by either choosing to explore novel, but anxiogenic spaces (open and brightly illuminated) or by retreating into safe (closed and dark) compartments. Widely accepted standards assume that the time spent in the less-anxiogenic, dark and protected areas reflects anxiety-like behaviour(Hascoët & Bourin 2009; Bourin et al. 2007). Statistical analysis revealed a significant interaction between sex*treatment, impacting the time spent in open arms as such that control males spent more time in open arms compared to control females and both Poly (I:C) sexes. Furthermore, a significant effect of sex on the "total ambulatory distance" in the LD was detected where females in both treatment

groups travelled further than males. No main effects of sex, treatment or interaction between the two factors for any other parameters, analyzed in the EPM and LD, were detected (see Table 2). The previous analysis on the effects of MIA in C57 mice has also yielded a significant difference in the time spent in open arms in the EPM between male offspring after MIA and vehicle controls, however, no modulatory effect of MIA on performance in the LD test has been revealed in C57 mice (Khan 2014).

Interestingly, other studies provide conflicting results concerning the effects of MIA on anxiety levels in C57 offspring, with one study yielding a significant impact of MIA and a modest effect of the factor sex on the time spent in closed arms in EPM (Hava et al. 2006) while others do not observe a significant difference in the anxiety-like behavior in adult MIA and control C57 offspring (Schwendener et al. 2009). However, type, dosage and timing of the immunogenic administration, together with sex and strain have to be considered as modulating variables, impacting the outcome of MIA on anxiety and depression (Babri et al. 2014). Indeed, sex and strain specific differences in anxiety response were previously described in other MIA animal models, and depend on timing of MIA, inflammation induction method and genetic background (Bolton et al. 2013; Paris et al. 2011; Babri et al. 2014). A sex specific difference in anxiety-like behavior can be hypothesized due to differential IL-1β responses between sexes, since augmented IL-1β levels have also been described in Poly (I:C) MIA mouse models (Brunton & Russell 2010; Paris et al. 2011; Hsiao & Patterson 2011). Hence it can be speculated, that differential cytokine levels in maternal and fetal compartments might relate to the significant differences in anxiety-like behavior between different sexes and treatment groups. Interestingly, a gross evaluation of anxiety-like behavior in the OF, which is reflected by the preference of the animal to remain and move in the periphery rather than the center of the arena, revealed a main effect of treatment for the parameter “center zone entries”, where Poly (I:C) offspring had more entries into the center compared to the control group, reflecting a reduction in anxiety-like behavior. However, all other characteristics thought to evaluate exploratory and locomotor activity were differentially modulated by either sex or treatment (see Table 2). In the preceding study, carried out in C57 mice, no effect of MIA, sex, or treatment*sex interaction was detected in the OF (Khan 2014), suggesting strain-specific sensitivity to the effects of MIA on the performance paralleling anxiety-like behavior in the OF. Interestingly, a comparative

study between three mice strains, including C57 and C3H mice, classified the C57 strain as "'non-emotional" while C3H were designated to be "emotional" mice (Kopp et al. 1999). This differential baseline level of emotionality, which is thought to reflect distinct levels of trait anxiety, was paralleled by strain-specific performances in the OF and LD tests (Kopp et al. 1999). However, while strain-dependent levels of emotional behaviors have also been reported by others, results obtained are not always comparable concerning the ranking of the degree of emotionality presented among the different inbred strains (Griebel et al. 2000).

The rota rod test, which evaluated motor coordination and motor learning, is routinely carried out as a control assay, to make sure that treatment-related alteration in motoric abilities cannot unspecifically confound the performance in other behavioral paradigms based on motor cooridnation (such as the FST).

Here, the latency to remain on the accelerating rotating drum is used as an indicator of motoric skills (Shiotsuki et al. 2010). Neither in C3H mice analyzed herein or in the previous report in C57 mice (Khan 2014), the latency to fall off the rod was different between the treatment groups or the sexes, indicating that MIA did not affect motor coordination. Interestingly, another study assessing the impact of genetic background and sex on the behavioral performance of transgenic mice did show important modulatory effects of both factors in several tasks, including the RR (Võikar et al. 2001).

Collective evaluations of present and previous results highlight the significance of the sex-dependent performance of mice in routinely used behavioral assays as well as its potential to interact with environmental factors constituting the experimental variable to be assessed in these tests. These observations are also extremely relevant considering the sex differences common in human diseases, regarding age of onset, disease progression or severity (Ober et al. 2008). This aspect is particularly relevant in regard to several psychiatric disorders, including depression, which show a clear distinction in prevalence between males and females (Seney & Sibille 2014), emphasizing that both sexes (males and females) should be tested when phenotyping mouse models of human diseases (Kovács & Pearce 2013). Female subjects are less frequently used in animal behavioral studies due to the varying hormonal levels in the different estrous phases which are known to modulate behavioral performance (Seney & Sibille 2014). However,

estrus synchronization and/or extension of sample sizes allow an independent evaluation of the various cyclic stages which could experimentally control the variability.

The results of maternal behavioral analysis revealed that the display of pup-oriented behavior and specifically the parameter LG of MB showed a significant difference between treatment and strain. Considering this, a modulation of depression-like behavior in the adult offspring was expected due to the known effect of varying levels of maternal care on behavioral performance of offspring later in life, mainly resulting from epigenetic mechanism (Champagne 2013). One potential factor underlying the strain dependent differences concerning the development of depression-like behavior induced by MIA, which is in agreement with previous reports using other experimental mouse models of adverse early life events (Schmidt et al. 2011), could be related to the specific genetic background potentially affecting the susceptibility of the development of depression-like behavior due to neurochemical differences among strains (Jacobson & Cryan 2007). Indeed, there are various relevant strain-dependent neurochemical distinctions which could affect the behavioral responsiveness, like regionally differentiated serotonin-dopamine interactions which directly relate to stress-induced anhedonia in different rodent strains (Bekris et al. 2005) and differential HPA axis responsiveness among distinct rodent strains (Wu & Wang 2010).

Another interesting aspect relates to the role of adult hippocampal neurogenesis in the subgranular zone of the dentate gyrus, which is highly implicated in depression and response to antidepressant therapeutic interventions (Santarelli et al. 2003; Dranovsky & Hen 2006; Samuels & Hen 2011; Mendez-David et al. 2013; Sahay & Hen 2007; Michael R Drew & Hen 2007) and has previously been shown to be impaired in adult offspring after Poly (I:C)-induced MIA (Khan et al. 2014). Interestingly, the inhibitory constraint of depressogenic environmental conditions, such as chronic mild stress, on the production of newborn neurons through the proliferation and differentiation of progenitor cell, has been reported to depend both on the strain and sex of experimental mice (Zhao et al. 2008; Kempermann et al. 1997). Along these lines, the stimulating effect of pharmacological antidepressants on the rate of proliferation and survival of newly born cells has also been found to differ among different inbred mouse strains (Holick et al. 2008). These results report strain-dependent sensivity of the behavioral

effects of chronic mild stress and response to treatments with various different classes of antidepressant drugs (Yalcin et al. 2008). Based upon these reports, a challenging hypothesis can be postulated in which the differential vulnerabilities of C3H and C57 mice to develop depression-like behavior as a consequence of the detrimental effects of Poly(I:C)-induced MIA could depend on the strain-dependent sensitivity of cellular processes related to adult hippocampal neurogenesis, differentiation of and survival on newly formed neurons. The rationale for this hypothesis which needs to be experimentally tested in future experiments, is further arising from a seminal study reporting that the amount and morphological characteristics of newly formed cells in the adult mouse hippocampus are modulated by maternal care behavior in a strain-dependent manner (Koehl et al. 2012). Results from this study suggest an interaction between an individual´s genetic make-up and the perinatal environment in shaping adult hippocampal neurogenesis, hereby possibly determining the susceptibility to the development of emotional disturbances throughout life. However, the molecular characteristics underlying this specific gene x environment interaction remain to be examined.

9.3 Hippocampal Corticosteroid Receptor Expression is Modulated by Sex, Strain and MIA-Treatment in Adult Offspring of C57 and C3H Mice.

Seeking to unravel some of the neurobiological mechanisms underlying the differential vulnerability of C57 and C3H mice to the effects of MIA on depression-like behavior later in life, we started out by examining the expression of hippocampal corticosteroid receptors as potential molecular interface mediating the interplay between MIA, maternal care and mood. To this end, hippocampal tissue from adult Poly (I:C)-induced MIA and control offspring (C57 and C3H mice) were used for qRT-PCR analysis examining levels of glucocorticoid (GR) and mineralocorticoid receptor (MR) expressions. 3-way ANOVA was used to determine the main effects of strain (C57 and C3H), sex (females and males) and treatment (saline and Poly (I:C)) and interaction between these terms. Significant effects in the interaction of sex*treatment and strain*treatment for GR mRNA levels and a significant strain*treatment interaction for MR expression were detected.

The interaction between strain*treatment for hippocampal GR and MR levels is particularly relevant considering the well-defined role of GR as major target of MB-induced epigenetic modifications, which is thought to be important for life-long changes in offspring stress vulnerability and dependent alterations in emotional behavior (Champagne 2013). Specifically, high levels of MB, especially LG, are known to increase hippocampal levels of GR hereby attenuate the HPA response to stress (Liu et al. 1997; Joseph Altman 2004; Francis et al. 1999). This effect is thought to be due to distinct levels of DNA methylation at GR gene promoter, facilitating access of transcription factors to chromatin regulatory proteins and a specific enhancement of histone acetylation at the GR locus, losing chromatin structure hereby favoring gene transcription (Meaney & Szyf 2005; Szyf 2009).

Here we observed that Poly (I:C) treatment had an effect on selected parameters of MB, most importantly on LG, which was independent of the mouse strain. However, depression-like behavior had only been detected in adult C57 but not C3H offspring. Considering these findings together with a strain*treatment interaction impacting hippocampal offspring GR and MR mRNA levels, these results invite postulating a possible involvement of the corticosteroid receptor system as the modulatory interface, determining the sensitivity of an organisms to the detrimental effects of MIA on depression-like behavior depending on the genetic background.

The sex*treatment interaction detected specifically for offspring hippocampal mRNA levels is highly relevant considering situation in the human population where mood disorders, including depression, are more prevalent in women than in men (Kessler et al. 1995; Brewin et al. 2000). This well-validated observation may relate to the known gender-differences in systemic (responsiveness of HPA axis) and cellular (MR and GR function) reaction to stress (ter Horst et al. 2012), also noticeable in the higher amounts of corticosterone secreted by females than males, both under baseline and under stress conditions (Critchlow et al. 1963; Carey et al. 1995; Figueiredo et al. 2002)

Importantly, corticosterone not only acts through GR but also binds with even higher affinity to MR, which is expressed more restrictedly than GR, namely mostly in the limbic system (McEwen et al. 1986; E. Ronald De Kloet et al. 1998; de Kloet et al. 2005), comprising brain areas involved in the generation and regulation of emotions. It is thought that MR is involved during the initial response of HPA axis while the GR concur

during the negative feedback of HPA axis by terminating the stress reaction, inducing recovery and behavioral adaption mechanisms (Oitzl & de Kloet 1992; Berger et al. 2006; E. Ronald De Kloet et al. 1998). Indeed, a dysbalance in the ratio of GR and MR expression has been proposed to be involved in the pathophysiology of stress-related psychiatric disorders, including depression (Berger et al. 2006; de Kloet et al. 2007).

Considering the strain-independent effect of Poly (I:C)-induced MIA together with the observation that the impact on depression-like behavior of adult offspring becomes only manifest in C57, but not C3H mice, it is tempting to speculate that the strain-specific effect of MIA on hippocampal GR and MR expression might constitute a molecular “relay station“ buffering the deleterious effects of MIA and altered MB in C3H mice. Following this hypothesis, one could propose that through hitherto unknown components of the genetic background and signaling pathways involved, the manifestation of (epigenetically transmitted) regulation of GR and MR expression as a result of dampened MB is compensated in the C3H strain, hereby preventing the development of the behavioral phenotype in the adult offspring. Hence, offspring hippocampal corticosteroid receptor expression could be considered as endophenotype, constituting the neurobiological interface conveying the impact of MIA induced alterations in MB on depression-like behavior later in life. However, this model including the possible epigenetic mechanisms involved, warrants further experimental assessment in future studies.

Alternatively, one has to consider MB, hippocampal GR and MR expression and the behavioral manifestations in adult offspring as associated, but not necessarily causally related consequences of MIA, all of which are to some extent dependent on the genetic composition of the experimental system (i.e. mouse strain) employed, as prominently known as confounding factor in (neuroscience) research (Bergink et al. 2014; Liu et al. 1997; Weaver, Cervoni, et al. 2004; Weaver, Diorio, et al. 2004; Danielle L Champagne et al. 2008; Oitzl et al. 2010; Ladd et al. 2004; Champagne 2008).

10 Conclusions and Perspectives for the Future

Taken together, the present study provides some important insights into the role of maternal immune activation in the pathogenesis of neuropsychiatric disorders, specifically depression, using the well-validated Poly (I:C)-MIA mouse model. Moreover, vital starting points for experimental investigations in imminent studies are provided.

First, the distinct and significant effect of Poly (I:C)-induced MIA on the characteristics of MB has been analyzed in detail, providing first evidence for a deleterious impact of MIA on maternal care in this model.

Second, the relevance of the genetic background for the impact of MIA on depression-like behavior in adult offspring has been demonstrated by the comparative evaluation in two different mouse strains. The precise genetic elements determining this strain-dependency remain to be elucidated.

Third, hippocampal corticosteroid receptor expression has been identified as a target of MIA in adult offspring brains, with its specific impact depending on the mouse strain, suggesting a potential relevance of modulation of GR and MR levels as endophenotype in this model. Possible (epigenetic) mechanisms accounting for the observed long-lasting changes in gene expression warrant future experiments in this direction. Moreover, the causal association and still missing molecular links between the events of Poly (I:C) stimulation - alterations in maternal care, hippocampal corticosteroid receptor expression and adult offspring behavioral manifestations - have to be examined.

The present study, together with impending future experiments, could provide an important leap forward in identifying the neurobiological basis of the effects of infections during pregnancy on offspring emotional behavior later in life and may by elucidation of the underlying molecular mechanisms involved and aid in the identification of potential new targets for the development of alternative therapeutic strategies in the treatment of depressive disorders.

11 References

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12 List of Abbreviations

ACTH	Adrenocorticotropic Hormone
ANOVA	Analysis of Variance
BDNF	Brain-Derived Neurotrophic Factor
C3H	C3H/HeNCrl
C57	C57BL/6N
CBT	Cognitive Behavioral Therapy
CMS	Chronic Mild Stress
CNS	Central Nervous System
CORT	Corticosterone
CRH	Corticotropin Releasing Hormone
D2R	Dopamine 2-receptor
E12.5	Embryonic day 12.5
ECT	Electroconvulsive Therapy
EPM	Elevated Plus Maze
ERα	Estrogen Receptor α
EtOH	Ethanol
$F_{(2,44)}$	Fisher-value(degrees of freedom)
F1	Filial1 generation
FST	Forced Swim Test
GR	Glucocorticoid Receptors
HPA	Hypothalamic-Pituitary Adrenal
HPG	Hypothalamic-Pituitary Gonadal
IDO	Indoleamine 2,3-dioxygenase
IL-1β	Interleukin-1β
IL-6	Interleukin 6
LD	Light Dark box
LG	Licking/Grooming
LPS	Lipopolysaccharide
MB	Maternal care Behavior
MDD	Major depressive disorder
MIA	Maternal Immune Activation
mPFC	Medial Prefrontal Cortex
MPOA	Medial Preoptic Nucleus
MR	Mineralocorticoid Receptor
mRNA	Ribonucleic Acid
n	number
NFkB-DNA	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells deoxyribonucleic acid
NMDA	N-Methyl-D-Aspartat
OF	Open Field
p	Probability value
P0	Postnatal day 0
Poly (I:C)	Polyinosinic:Polycytidylic Acid
PRS	Prenatal Restraint Stress
PVN	Paraventricular Nucleus
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
RPM	Revolutions per Minute
RT	Room Temperature
SEM	Standard Error of Mean
SNRIs	Selective Norepinephrine Reuptake Inhibitors
SPT	Sucrose Preference Test
SSRIs	Selective Serotonin Reuptake Inhibitors
TLR-3	Toll-like receptor 3
TLR-4	Toll-like receptor 4
TMS	Transcranial Magnetic Stimulation
TNF-α	Tumor Necrosis Factor α
TRYCATs	Tryptophan Catabolites
TST	Tail Suspension Test
ΔCT	Difference Cycle Threshold

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Publications

Savalli G, Diao WF, **Berger S**, Ronovsky M, Partonen T, Pollak DD. Anhedonic behavior in cryptochrome 2-deficient mice is paralleled by altered diurnal patterns of amygdala gene expression. *Amino Acids, in press* (DOI 10.1007/s00726-015-1968-3)