Developmental and psychoneuroimmunological mechanisms in depression

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Developmental and psychoneuroimmunological mechanisms in depression

Yara Pujol López

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Developmental and psychoneuroimmunological mechanisms in depression

DISSERTATION

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in accordance with the decision of the Board of Deans,

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by

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Pels meus avis Joan i Josefina,
para mis yayos Carmen y Luis
y para mi ahijada Irina.

Contents

Chapter 1	9
General Introduction	
Chapter 2	25
Prenatal maternal infection and offspring brain development: programming of adult psychopathology	
Chapter 3	71
Effects of subcutaneous lipopolysaccharide (LPS) injection on gestational length and intra-uterine and neonatal mortality in mice	
Chapter 4	87
Quinolinic acid (QUIN)-immunoreactivity in the naïve mouse brain	
Chapter 5	111
Effects of prenatal polyinosinic:polycytidylic acid (Poly I:C) exposure on global histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity in the mouse brain	
Chapter 6	133
Depressive symptoms during interferon-alpha (IFN- α) therapy in humans associated with changes in the kynurenine pathway	
Chapter 7	163
General Discussion	
Summary	181
Valorization	187
Acknowledgements	197
Curriculum Vitae	217
Publications	219

Chapter 1

General introduction

Yara Pujol López

Background

The expression of psychiatric disorders includes a wide range of different categories of afflictions that are currently classified by two widely established systems: in the International Classification of Diseases (ICD-11) formulated by the World Health Organization (WHO) and in the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) formulated by the American Psychiatric Association (APA) (Cuthbert and Insel 2013).

Among psychiatric disorders, major depression is the leading cause of disability (WHO 2012) comprising several symptoms, such as sadness, anxiety, hopelessness, loss of interest, fatigue, and insomnia (Health 2010). To recognise the large impact of depression in our society, it is reported that currently more than 350 million people are estimated to suffer from depression including an increasing number of young persons (WHO 2012). Apart from the increasing number of people suffering from depression and the devastating effect on the quality of life of patients, the disease is associated with a high social, economic and societal burden. Depression is associated with divorce and suicide, among others (Wolfersdorf 2008). In addition, people suffering from depression reduce their productivity in the workplace, which has a considerable economic impact (Gilbody, Bower et al. 2012, McTernan, Dollard et al. 2013).

Diagnosis and treatment of major depression

Nowadays, the diagnosis of major depression is still based on a clinical psychiatric interview, complemented with specific questionnaires based on

ICD-10 or DSM-V criteria. Suitable biomarkers for depression are still missing. The subjective nature of diagnosing depression is further complicated by patient-specific behaviour: the patient may or may not collaborate and often describes his/her symptoms in a personal manner and the doctor needs to be able to understand and interpret them. Moreover, a diagnosis is often difficult to make because of the many different ways in which clinical depression can be manifested.

Regarding treatment, it is essentially based on psychotherapy and the use of antidepressants. Since the 1950s, different generations of antidepressants were launched on the market with the purpose of increasing several neurotransmitter levels, involving serotonin (5-HT) and noradrenaline (NA) among others. However, to date, no significant improvement in the rates of relapse has been observed after using different therapeutic options. Some patients do not even respond to any form of treatment. Others do, but often show recurrence. Altogether, there is a high need to develop more effective treatment options, which requires more insight in the molecular mechanisms underlying this debilitating disorder.

Hypotheses on the pathogenesis of depression

As from the beginning of the last century, several hypotheses have been suggested to explain the pathogenesis of depression. Although they are addressed in detail in Chapter 2, an overview of the hypotheses is provided below.

First, the monoamine hypothesis was proposed, associating depression to reduced levels of monoamines, such as 5-HT and NA (Schildkraut 1995). This

theory was supported by the fact that the serendipitously discovered antidepressants such as tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors (MAOIs) increase synaptic monoamine levels, and dominated the field of depression research for several decades. However, this hypothesis could not explain why medication that increases monoamine levels is not effective for all depressive patients, and it neither clarified the pathophysiology of depression (Hirschfeld 2000, Hindmarch 2002). In addition, changes in monoamines have not been consistently described and there is a delay between the moment when antidepressants are administered and after which clinical effects are observed (Stahl 2000). Altogether, it suggests that other mechanisms mediating the monoamine system may be involved, such as changes in receptors or in epigenetic mechanisms. As such, alternative theories were formulated. The hypothalamic-pituitary-adrenal (HPA) axis or corticosteroid hypothesis of depression suggests that hyperactivity of the HPA axis and concomitant high levels of corticosteroids may be involved in the development of depression. Hyperactivity of the HPA axis is indeed often observed in depressed patients (Swaab, Bao et al. 2005) and antidepressants are known to induce changes in the levels of glucocorticoid receptors (GR), which sustains this hypothesis (Anacker, Zunszain et al. 2011). Moreover, evidence shows increased production and secretion of glucocorticoids in depressed patients (Gillespie and Nemeroff 2005) and impairment of the GR, e.g. glucocorticoid resistance and concomitant alteration of the feedback regulation of the HPA axis (Pariante and Miller 2001). Another theory links depression with a deficit in neurotrophic factor signaling. Specifically, studies focusing on

brain-derived neurotrophic factor (BDNF) showed hippocampal atrophy and reduced serum BDNF levels in depressed patients. Interestingly, a decrease in BDNF levels was reversed after antidepressant treatment (Shimizu, Hashimoto et al. 2003, Gonul, Akdeniz et al. 2005, Yoshimura, Mitoma et al. 2007). In addition, antidepressants have been reported to normalize BDNF levels in the hippocampus in postmortem brain tissue of patients suffering from major depression (Chen, Dowlathshahi et al. 2001).

The immune system has also been related to depression, initially formulated in the macrophage theory of depression. According to this hypothesis, excessive secretion of cytokines by macrophages and monocytes would cause depression (Smith 1991). Several studies corroborate this theory showing increased inflammatory markers in some depressive patients (Maes 1999, Howren, Lamkin et al. 2009). For instance, evidence shows increased interleukin (IL)-6, C-reactive protein and tumor necrosis factor alpha. (TNF- α) (Dinan 2009). In addition, as a comorbid illness, major depression is prevalent in cancer (Snyderman and Wynn 2009) and cardiac patients (Huffman, Celano et al. 2013), disorders often associated with inflammation. Moreover, administration of interferon-alpha (IFN- α), a pro-inflammatory cytokine commonly used to treat hepatitis C, is reported to induce major depression in up to 45% of hepatitis C patients (Asnis and De La Garza 2006). Interestingly, anti-inflammatory drugs are able to alleviate depression in some patients (Muller 2013), and commonly used antidepressants show anti-inflammatory properties, such as a reduction in pro-inflammatory cytokines (Kim, Suh et al. 2002, Cavanagh and Mathias 2008). Following the link between inflammation and depression, the cytokine hypothesis of

depression was formulated. This hypothesis suggests cytokines as the key mechanism responsible for inducing depressive behaviour via an increased degradation of tryptophan (TRP) to neurotoxic catabolites along the kynurenine pathway (Maes 2008). First TRP can be metabolised to 5-HT by tryptophan hydroxylase (TPH) or to kynurenine by tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) (Leonard and Myint 2009). TDO is mainly located in the liver, whereas IDO has been detected in many tissues, such as brain, placenta, pancreas, among others (Moffett and Namboodiri 2003). Kynurenine is further metabolised by kynurenine aminotransferases (KATs) to kynurenic acid (KYNA), which acts as a N-methyl-D-aspartate glutamate (NMDA) glutamate receptor antagonist in the brain. Alternatively, kynurenine can be metabolised to 3-hydroxykynurenine (3HK) by kynurenine-3-monooxygenase (KMO), after which it is further degraded to 3-hydroxyanthranilic acid (3HAA) by kynureninase (KYNase). 3HAA is involved in the synthesis of adenosine triphosphate (ATP), mainly in the liver, and degraded into quinolinic acid (QUIN) by 3-hydroxyanthranilic acid oxygenase (3HAO). QUIN is a NMDA glutamate receptor agonist and can be degraded into nicotinamide adenine dinucleotide (NAD) (Sperner-Unterweger, Miller et al. 2002, Myint 2012) (see Figure 1). Interestingly, various enzymes involved in the kynurenine pathway can be modulated by cytokines. For instance, both interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) activate IDO and KMO, whereas TDO activity can be further enhanced by glucocorticoids (Myint 2012). Thus, activation of the kynurenine pathway will lead to depletion of TRP, and consequently of 5-HT. In addition, KYNA is known as a

potential neuroprotective metabolite, whereas QUIN and 3HK induce neurotoxic effects, such as excitotoxic neuronal cell loss and convulsions (Nemeth, Toldi et al. 2005). Along this line, the neurodegeneration hypothesis of depression was formulated which integrates the above mentioned theories, the kynurenine pathway and its connection with cytokines, the kynurenine metabolites, 5-HT as well as NMDA-glutamatergic neurotransmission (Myint and Kim 2003). Specifically, this hypothesis emphasizes the importance of a balance between kynurenic and quinolinic acid, considered as a neuroprotective and neurotoxic metabolites respectively.

Figure 1. Schema of tryptophan metabolism. See text for details. Abbreviations: TPH, tryptophan hydroxylase; 5-HT, serotonin; GC, glucocorticoids; IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan 2,3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine-3-monooxygenase; KYNase, kynureninase; 3HAO, 3-hydroxyanthranilic acid oxygenase; QPRT, quinolinic acid phosphoribosyl transferase; NAD, nicotinamide adenine dinucleotide.

Prenatal maternal infection and depression in the offspring

Several insults during pregnancy have been related to psychopathology in the offspring (Weinstock 2001, Zammit, Thomas et al. 2009). Interestingly, inflammatory processes have been suggested as key mediators underlying the relationship between prenatal environmental insults and the development of psychiatric disorders later in life (Patterson 2002, Meyer, Yee et al. 2007). As such, several animal studies have been performed in order to investigate the link prenatal maternal infection and offspring psychopathology (Beumer, Gibney et al. 2012), although they particularly focused on schizophrenia (Zuckerman, Rehavi et al. 2003, Ozawa, Hashimoto et al. 2006, Meyer and Feldon 2009). The exact mechanisms that mediate the relation between prenatal maternal infection and depression in the offspring remain to be elucidated. Epigenetic processes likely candidate mechanisms in this respect.

Epigenetic mechanisms

The term epigenetics describes heritable changes in gene expression that do not involve alterations of the DNA sequence. The most-studied epigenetic mechanisms to date involve DNA methylation and histone modifications, which are addressed below.

DNA methylation implies the methylation of cytosine in DNA forming 5-methylcytosine (5mC). The enzymes responsible for this process are DNA methyltransferases (DNMTs). DNMT1 mediates the conservation of methylation during DNA replication, whereas DNMT3 regulate *de novo*

methylation. DNA methylation is involved in the regulation of gene expression by methylation of CpG dinucleotides and by methyl CpG-binding domain (MBD)-containing protein family members. Generally, DNA methylation results in the suppression of gene expression (Das and Chai 2013).

Histones are proteins that together with the DNA and other proteins form the chromatin, whose basic unit is the nucleosome. A nucleosome consists of 2 of each classical histone proteins (H2A, H2B, H3 and H4) wrapping 147 base pairs of DNA. Post-translational modifications in the tail of these histones, such as acetylation, deacetylation, methylation, phosphorylation, ubiquitylation and others, affect chromatin structure. The concomitant changes in compaction of the chromatin modulate interactions between DNA sequences and transcription factors, DNA binding proteins, ncRNAs, thus altering gene expression (Das and Chai 2013). The most common modifications are histone acetylation and deacetylation, regulated by histone acetyltransferases (HAT) and histone deacetyltransferases (HDAC), respectively. Acetylation of histones results in heterochromatin, which facilitates the access and binding of transcription factors and additional nuclear proteins, promoting transcription. On the contrary, deacetylation of histones leads to compacted euchromatin and transcriptional repression.

Interestingly, infections can modulate epigenetic processes in many ways (Bierne, Hamon et al. 2012), whereas epigenetic regulation is known to target genes involved in cell progression, survival, inflammation and immunity. Thus, being a key player in the development and differentiation of the nervous system that may explain the response of the central nervous

system (CNS) at a molecular level, in case of infection and concomitant injury.

Aim and outline of this thesis

The overarching aim of this thesis is to investigate the intricate role of inflammation in depression. More specifically, we hypothesized that alterations in the kynurenine pathway are causally involved in mediating changes in inflammation-induced depression-related phenotypes. Moreover, we postulated epigenetic changes as mechanisms underlying the association between prenatal inflammation and adult psychopathology.

In **Chapter 2** of this thesis we review evidence on how exposure to prenatal maternal infection may interfere with offspring brain development. Moreover, sex-differences in the response to prenatal maternal infection as well as treatment options are discussed.

Chapter 3 highlights how prenatal infection using an endotoxin contained in bacterial cell walls, lipopolysaccharide (LPS), affects gestational length and intra-uterine and neonatal mortality in mice.

In **Chapter 4**, we provide an analysis of QUIN in the naïve mouse brain in relation to affect-related behavior.

Chapter 5 examines alterations in epigenetic mechanisms, such as global HDAC and DNMT activity in the mouse brain after prenatal exposure to polyinosinic:polycytidylic acid (Poly I:C).

In **Chapter 6**, we explore alterations in diverse enzymes and metabolites of the kynurenine pathway in relation to depression scores of IFN- α treated patients.

Finally, **Chapter 7** provides a general conclusion for our findings.

Of note, for our mouse studies, we used the C57BL/6JRccHsd mouse strain (Harlan Laboratories, Germany). This is a well characterized mouse strain frequently used in behavioral research. C57Bl/6J mice show low 'emotionality', high open-field exploration, high locomotor activity and only short time of immobility during the forced swim test (Thompson 1953, Nikulina, Skrinskaya et al. 1991). This specific strain was also chosen because they still carry the gene that encodes for the enzyme nicotinamide nucleotide transhydrogenase, which is missing in the original C57BL/6 strain. This enzyme is important for our field of research because it is involved in the last reaction of the kynurenine pathway, where it converts NAD⁺ to NADP⁺ which in turn is used for biosynthesis and detoxification. In addition, this strain does not lack α -synuclein, which belongs to a family of structural proteins which is highly expressed in the brain. For these reasons, the C57BL/6JRccHsd strain is more wild-type like, which is important to ensure that brain morphology and functioning is not affected by any of these genetic mutations.

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Chapter 2

Prenatal maternal infection and offspring brain development: programming of adult psychopathology

Yara Pujol Lopez; Daniel LA van den Hove; Harry WM Steinbusch; Gunter Kenis; Aye M Myint

Manuscript in preparation

Abstract

This review addresses the possible effects of inflammation during pregnancy and its impact on offspring brain development. Activation of the immune system in pregnant women by e.g. infection or stress can result in different degrees of inflammatory responses, which in the long run may lead to the development of psychiatric disorders in the offspring. More specifically, during normal pregnancy, increased gonadal hormone levels induce immunosuppressive effects, through which women protect their foetus. This state of immune tolerance can be disrupted by the trigger of an inflammation, thereby impacting upon foetal brain development, concomitant with an increased predisposition to mental disorders in adulthood. As part of this process, maternal infection-induced cytokine production affects the action of enzymes, neurotransmitters, and hormones, a disturbance of which may have a lasting impact upon offspring brain function and behaviour. In addition, the kynurenine metabolism, in which the essential amino acid tryptophan is catabolised through several steps to quinolinic and kynurenic acid, has been shown to be affected in response to inflammation. Both metabolites are reported to regulate the balance between neurodegeneration and neuroprotection in the brain. Furthermore, a disturbance of the kynurenine metabolism also affects the availability of tryptophan, reducing the synthesis of serotonin. Moreover, part of the programming effects induced by inflammation during pregnancy is hypothesized to be mediated by epigenetic mechanism. As such, the present review will discuss the link between prenatal maternal infection, epigenetic processes, neuro-immune interactions and neurodegenerative

26

damage to the central nervous system in view of the development of psychiatric disorders in the offspring.

1. Introduction

Over the last decades, advances in psychiatric research have led to the notion that inflammatory processes and neuro-immune interactions are involved in the pathogenesis of mental disorders such as depression (see review by Gibney and Drexhage 2013). For example, evidence has shown that some individuals develop depression after exposure to infectious agents (see review by Dantzer, O'Connor et al. 2008, Benros, Waltoft et al. 2013), such as the Hepatitis C virus (see review by Zdilar, Franco-Bronson et al. 2000, Carta, Hardoy et al. 2007). Along similar lines, exposure to different pathogens during pregnancy, by impacting upon brain development and behaviour, has been shown to increase the risk for the development of psychiatric disorders in the offspring (see review by Boksa 2010). For instance, an elevated risk for the development of bipolar disorder, schizophrenia and autism has been observed in offspring of women who were infected with bacteria or viruses during gestation (Brown 2006, Atladottir, Thorsen et al. 2010, Parboosing, Bao et al. 2013).

To further investigate which mechanisms mediate the relation between infection during pregnancy and an increased risk to develop psychiatric disorders in the offspring, several studies have been performed using a variety of animal models, particularly in rats and mice. Examples include prenatal maternal exposure of rodents to lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (Poly I:C) or the influenza virus. Both in response to maternal LPS and Poly I:C exposure rat offspring showed a deficit in prepulse inhibition (PPI) (Borrell, Vela et al. 2002, Wolff and Bilkey 28

2008), which assesses the ability to gate sensory information, a reduction of which is observed in schizophrenia. In addition, after exposing pregnant mice to Poly I:C, memory deficits were observed in the offspring when subjected to the Morris water maze and novel object recognition test (Ozawa, Hashimoto et al. 2006, Meyer, Nyffeler et al. 2008). The exact mechanisms underlying brain dysfunction in offspring exposed to prenatal maternal infection remain to be elucidated though. This article provides a literature review about the link between prenatal maternal inflammation and the development of psychiatric disorders in the offspring and the possible mediators involved, including cytokines, neurotransmitters, neurotrophins and hormones as well as related epigenetic processes.

1.1. Psychoneuroimmunology of depression.

In the 90s of the last century, numerous studies reported immunological abnormalities observed in depressed subjects. Some studies found an increase of plasma pro-inflammatory cytokines levels (Interleukin [IL]-1, IL-6 and IL-8) as well as C-reactive protein (CRP) in depressed patients (Song, Lin et al. 1998, see review by Shelton and Miller 2011), whereas another study reported decreased levels of IL-1b and IL-2 when comparing healthy controls and depressive patients (Weizman, Laor et al. 1994). Several studies reported a reduction in NK cell toxicity, relative leucocytosis, slightly increased CD4/8 ratios, i. e. activation of cellular immunity, and reduced IL-6 levels in the cerebrospinal fluid (CSF) of patients with major depression (Stubner, Schon et al. 1999, see review

by Zorrilla, Luborsky et al. 2001). On the other hand, other studies showed an increase in the number of cells with activation markers, in positive acute phase proteins, i.e. produced by the liver, induced by IL-6, and in the synthesis of prostaglandins and circulating soluble IL-2 receptors (see review by Maes 1999, Sluzewska 1999). Moreover, a decrease in negative acute phase proteins (Maes, Scharpe et al. 1992) and an overproduction of cytokines, such as the pro-inflammatory cytokines IL-1, IL-6 and IFN, have been reported (Maes, Smith et al. 1995). These levels of acute phase proteins, i.e. an increase in positive and a decrease in negative acute phase proteins are considered to be indicative of an inflammatory state (see review by Dunn, Swiergiel et al. 2005). Moreover, depression is known to be induced after administration of pro-inflammatory cytokines, such as IFN- α when treating an infectious disease or cancer (Raison, Capuron et al. 2006). Antidepressants have also been shown to possess anti-inflammatory and neuroprotective properties, by reducing pro-inflammatory cytokines (see review by Shelton and Miller 2011). Given the amount of evidence showing inflammation in some depressed patients, an inflammatory hypothesis of depression was first proposed by Smith (1991). This hypothesis is known as the “macrophage theory of depression” which related depression to immune activation (Smith 1991). To combat infection, fever and physiological changes in the body occur, concomitant with adaptive behavioural alterations. Cytokine activation, particularly of the pro-inflammatory type, regulates various hormones inducing behavioural alterations associated with the induction of sleep,

anhedonia, irritability, etc., which, as a whole, is defined as “sickness behaviour” (Raison, Capuron et al. 2006). Depression and sickness behaviour have several symptoms in common including anhedonia, decreased appetite and activity, and disturbed sleep (see review by Hashmi, Aftab et al. 2013). This notion led to the “cytokine hypothesis of depression”, which suggests a causal role for cytokines in inducing depressive behaviours by an increased metabolism of tryptophan to neurotoxic catabolites along the kynurenine pathway (Maes 2008).

2. Pregnancy and the immune system

2.1 The immune system during normal pregnancy

Classically, pregnancy has been regarded as a state of immune suppression in order to protect the foetus. However, over the last years, increasing evidence has shown that during gestation the immune system, though tightly regulated, is in fact very active (see review by Mor and Cardenas 2010). Generally, three different inflammatory phases are observed during pregnancy. First, during embryo implantation, placentation and the early stages of pregnancy, a pro-inflammatory environment dominates, whereas during mid-pregnancy an anti-inflammatory milieu is observed, returning to a pro-inflammatory one at the third trimester and the end of pregnancy (see review by Amirchaghmaghi, Taghavi et al. 2013). A strong inflammatory response is required during the first phase of pregnancy to break the epithelium of the uterus stimulating the process of implantation and subsequent

steps including the invasion and replacement of endometrial tissue (see review by Dekel, Gnainsky et al. 2010). Second, a shift to an anti-inflammatory allows a rapid foetal growth and development (see review by Mor and Cardenas 2010). Finally, a pro-inflammatory state is restored in order to stimulate the contraction of the uterus, delivery of the baby and rejection of the placenta (Mor 2008).

Along pregnancy, increased numbers of peripheral white blood cells have been detected in the peripheral maternal immune system (Veenstra van Nieuwenhoven, Bouman et al. 2002). In human decidua, elevated numbers of immune cells, e.g. natural killer (NK) cells, macrophages and regulatory T cells (Treg) are present during normal pregnancy (Bulmer, Pace et al. 1988, see review by Mor and Cardenas 2010). Specifically, NK cells were found to be increased during the first trimester of pregnancy (Hidaka, Amino et al. 1991). However, macrophages were elevated in early and in late gestation as well (Bulmer, Pace et al. 1988). Regarding Treg cells, an increase has been observed during both the first the second trimester of pregnancy (Somerset, Zheng et al. 2004).

2.2 Infection during pregnancy and inflammatory changes during gestation

As already mentioned, the exposure to an infectious agent during pregnancy is a major risk factor for neurodevelopmental brain damage (Golan, Lev et al. 2005). Maternal infection induces the production of cytokines and glucocorticoids (GCs), which have been hypothesized to

be critically involved in mediating the development of e.g. neurodevelopmental disorders in the offspring (see review by Zhou 2012). Cytokines, such as IL-1B, IL-6 and tumor necrosis factor- α (TNF- α) are induced in maternal and/or foetal tissues, with maternal cytokines being able to cross the placenta and to affect the development of the brain via different mechanisms (Ashdown, Dumont et al. 2006) for instance, through their direct involvement in brain development, such as lineage commitment, differentiation and survival of neurons and glial cells (see review by Zhou 2012). Furthermore, at a systemic level, cytokines represent key regulators of the inflammatory response triggering the immune response, provoking fever and initiating an elevated synthesis of acute phase proteins, whereas locally, they help to recruit inflammatory cells to inflammation sites (see review by Marques, Cizza et al. 2007). Moreover, cytokines are implicated in the modulation of a variety of processes in chemical synapses, such as neurotransmitter synthesis, storage, release, (re)uptake, degradation and interacting with specific neurotransmitter receptors as well as related signal transduction cascades, which are crucial in the regulation of CNS function (see review by Camacho-Arroyo, Lopez-Griego et al. 2009). Finally, they have also been shown to be involved in placental dysfunction and apoptosis of the trophoblast (see review by Boksa 2010).

Next to cytokines, GCs are known to represent key players mediating the effects of prenatal maternal infection. GCs which are considered immunosuppressors that play a vital role in the control of the immune response (see review by Zhou 2012), are generally produced after

activation of the hypothalamo-pituitary adrenal (HPA) axis, particularly in response to stress. In addition, maternal infection is known to result in elevated level of maternal blood GCs, concomitant with elevated foetal GC levels (see review by Zhou 2012). The concomitant effects of GCs are commented in detail below and in the section reviewing the stress and its involvement in depression.

To sum up, various factors impact upon the CNS in offspring exposed to prenatal maternal infection, including cytokines, stress hormones and neurotransmitters.

2.2.1 Infection during pregnancy and the neuroimmune system axis

2.2.1.1 The corticosteroid hypothesis of depression

This hypothesis postulates that chronic and/or excessive stress exposure and concomitant stress hormone dysregulation represent a major cause for developing depression. In view of inflammation, interestingly, there is evidence of increased HPA axis activity after infection (see review by Kronfol and Remick 2000) and of modulation of the axis by cytokines (Raison, Capuron et al. 2006). GCs control the secretion of corticotropin-releasing factor (CRF) and adrenocorticotrophic hormone (ACTH) executing a negative feedback at the level of the hypothalamus and pituitary, by activating the glucocorticoid receptor (GR) (see review by Leonard and Myint 2009). GCs like cortisol generally act as immunosuppressors via e.g. inhibition of IL-1, IL-2 and IL-6 production or secretion, depletion of

CD4+ T cells, or promoting cell death (Maes, Smith et al. 1995). However, their effects on the immune system vary temporarily. Under physiological conditions they might enhance pro-inflammatory cytokines, whereas during the acute phase of the immune response, they are potent inhibitors of the synthesis and actions of central and peripheral cytokines (see review by Howerton and Bale 2012). Moreover, GCs produce different effects depending on the tissue type. For example, elevated GCs suppress the peripheral immune system, whereas they promote a pro-inflammatory state in immune cells in the brain (Howerton and Bale 2012). Some cytokines, such as TNF- α , IL-1 and IL-6 can directly activate the HPA axis inducing the hypothalamic release of CRF, and consequently ACTH and GCs (Rook, Hernandez-Pando et al. 1994). Cytokines can also exert direct effects on the pituitary and adrenal glands. However, peripheral cytokines can affect the HPA axis by other, more indirect ways too. For example, peripheral injection of IL-1 stimulates the hypothalamic release of noradrenaline that in turn stimulates the release of CRF (Dunn 1988). In addition, Th1 and pro-inflammatory cytokines are reported to decrease GR affinity, which contributes to the vicious circle of immune activation. These actions, together with impaired HPA axis control of cytokine secretion, are decisive in mediating disease progression (see review by Irwin 2002). In relation to pregnancy, GCs are able to pass the placenta (Edwards, Benediktsson et al. 1993) and exposure to normal physiological levels of GCs is in fact required for e.g. foetal

development (Kitraki, Kittas et al. 1997), regulating brain processes such as subcellular reorganization of neuron-neuron and neuron-glia interactions (see review by Matthews 2000). Importantly, various mechanisms exist to prevent excessive exposure to GCs. For example, at the placenta, the enzyme 11 β hydroxysteroid dehydrogenase 2 (11 β HSD2) can convert GCs to inactive metabolites (Seckl 1997), thus protecting the developing foetus in case of elevated exposure to GCs, which could lead to alterations in the neurodevelopment of the foetus (see review by Zhou 2012). Decreased placental 11 β HSD2 activity or expression has been associated with elevated GCs exposure in the foetus and birth complications in human and animals model (see review by Howerton and Bale 2012). In view of prenatal infection, rats prenatally exposed to LPS showed increased stress response, concomitant with elevated levels of anxiety-like behaviour. Moreover, after exposure to stress, offspring of LPS-injected dams, showed elevated corticosterone responses and reduced GR levels in the dorsal hippocampus (Lin, Lin et al. 2012). In offspring of rats injected with Poly I:C during pregnancy, stress-induced corticosterone levels were reduced compared to controls (Maayan, Ram et al. 2012). Other studies investigated corticosterone levels in rodents in both mothers and offspring after prenatal exposure to LPS. Whereas, one group reported increased levels of corticosterone in both pregnant dams and male offspring exposed to LPS (Enayati, Solati et al. 2012), other studies only found elevated maternal corticosterone levels and no

changes in the offspring in response to prenatal maternal LPS exposure (Asiaei, Solati et al. 2011, Kirsten, Lippi et al. 2013). These differences may potentially be explained by the different timing of LPS injections during pregnancy and the different age of the offspring when studied.

2.2.1.2 Neurotrophic hypothesis of depression

The formulation of this hypothesis is based on the observation of reduced levels of neurotrophins like brain-derived neurotrophic factor (BDNF) in response to stress and in stress-related disorders like major depression, as well as the beneficial effects of antidepressants in this respect, e.g. by upregulating BDNF. Neurotrophic factors mediate neuronal survival, a wide range of neuronal and glial cell activities, e.g. axonal, dendritic growth, synaptic structure and plasticity, neurotransmitter expression, long-term potentiation, and various immune cell functions, such as immune cell activation, differentiation, migration, and local antigen presentation (Hohlfeld, Kerschensteiner et al. 2007). An additional function is the inhibition of neuronal death or degeneration. In fact, its levels and receptors are vigorously regulated in pathological conditions, which suggest the importance of neurotrophic factors in the response of neurons to degenerative or traumatic processes. They also regulate various important elements of inflammation, such as the migration of monocytes across the blood-brain barrier (BBB) and the release of pro-inflammatory cytokines (see review by Kerschensteiner,

Stadelmann et al. 2003). The most-studied neurotrophic factor is BDNF, which is known to play an important role in regulation of neuronal survival, development and function (see review by Walker, Kim et al. 2014). Increased evidence reported the involvement of BDNF the pathogenesis of major depression as well as in mediating the action of antidepressants. For instance, reduced expression of its receptor (TrkB) has been observed in postmortem brain samples of depressed patients (Tripp, Oh et al. 2012). In addition, reduced levels of mature BDNF, but not of its precursor were shown in sera from patients suffering from depression (Karege, Perret et al. 2002, Yoshida, Ishikawa et al. 2012). After using antidepressants, elevated serum BDNF levels have been reported (Yoshimura, Mitoma et al. 2007). In view of infection during pregnancy, discordant results on BDNF have been reported. Offspring of pregnant rodents injected with LPS showed elevated brain BDNF levels (Gilmore, Jarskog et al. 2003, Golan, Lev et al. 2005), which may be explained as a consequence of a regenerative response to LPS. However, in another study, rat offspring prenatally exposed to LPS showed reduced BDNF in the hippocampus on postnatal day 21 and 90 (Lin and Wang 2014). When rats were exposed to *Escherichia coli* during gestation, increased expression of hippocampal BDNF and TrkB were observed in the offspring at postnatal 3 and 7 days (Jiang, Zhu et al. 2013).

2.2.1.3 Monoamine hypothesis of depression

During the 1950s and 1960s of the last century, the monoamine hypothesis led research on psychiatric disorders. This hypothesis is centred on the impaired levels and activity of monoamines, particularly serotonin (5-hydroxytryptamine or 5-HT), in the development of depression (see review by Schildkraut 1965, Coppen 1967). Indeed, reduced blood 5-HT levels (Takahashi 1976) and reduced brain 5-HT transporter (5-HTT) availability (Willeit, Praschak-Rieder et al. 2000) have been found in patients suffering from depression. Besides 5-HT, reductions in brain dopamine levels (Martinot, Bragulat et al. 2001) and downregulation of the norepinephrine transporter (NET) have been shown in depressed patients (Klimek, Stockmeier et al. 1997). However, administration of antidepressants that increase monoamine levels, are not always effective (see review by Shelton 2006), indicating that this concept is oversimplified.

A closely related hypothesis, initially formulated in 1969, suggested the depletion of tryptophan, essential amino acid and precursor to 5-HT due to an increased kynurenine synthesis as the cause for major depression. 5-HT is synthesized from the essential amino acid tryptophan, which is actively transported into the brain through the BBB (Fernstrom 1984). As such, the synthesis of 5-HT depends on the plasma levels of tryptophan (Song, Lin et al. 1998). However, this essential amino acid can be metabolised by two different pathways: to 5-HT by the enzyme tryptophan hydroxylase (TPH) and to

kynurenine by tryptophan 2,3-dioxygenase (TDO), mainly in the liver (Watanabe, Fujiwara et al. 1980), and by extrahepatic indoleamine 2,3-dioxygenase (IDO), e.g. in the brain (Moffett, Blinder et al. 1998). Whereas TDO is specific for tryptophan, IDO is also able to metabolize other substrates such as 5-HT and melatonin (Hayaishi 1976, Dang, Dale et al. 2000). Furthermore, TDO regulates whole basal serum tryptophan levels, IDO is up-regulated as a result of inflammatory conditions (Niinisalo, Raitala et al. 2008). In addition, TDO is stimulated by cortisol, whereas IDO is induced by pro-inflammatory cytokines, such as IFN- γ , IL-6 and TNF α (see review by Maes, Leonard et al. 2011), and suppressed by anti-inflammatory cytokines (see review by Leonard and Myint 2009), such as IL-4 and IL-10 (Weiss, Murr et al. 1999).

Both TPH and IDO activity are directly and indirectly correlated with immune activation (Sperner-Unterweger, Miller et al. 2002). Whereas in physiological conditions tryptophan is metabolised mainly by TDO, an immune challenge suppresses TDO and stimulates IDO. IDO controls the quantity of tryptophan that will be metabolised to kynurenine (Maes, Meltzer et al. 1993) and increased levels of IDO have been associated with increased anxiety and impaired mood (see review by Maes 2011). In particular, two important metabolites, i.e. quinolinic acid (QUIN) and kynurenic acid (KYNA) are synthesised through the kynurenine pathway. Kynurenine is further metabolised to KYNA by kynurenine aminotransferases (KATs). Alternatively, kynurenine is metabolised to 3-hydroxykynurenine (3HK) by

kynurenine-3-monooxygenase (KMO), after which it is further degraded to 3-hydroxyanthranilic acid (3HAA) by kynureninase. 3HAA is involved in the synthesis of adenosine triphosphate (ATP), mainly in the liver, and degraded into QUIN by 3-hydroxyanthranilic acid oxygenase (3HAO). QUIN is then degraded into nicotinamide adenine dinucleotide (NAD) (Sperner-Unterweger, Miller et al. 2002, see review by Myint 2012) (see Figure 1 in the introduction chapter). Both QUIN and KYNA directly mediate glutamate neurotransmission (Bay-Richter, Janelidze et al. 2011). These metabolites represent intercellular messengers with neuroactive, proapoptotic and immunoregulatory properties (see review by Zelante, Fallarino et al. 2009). Whereas KYNA is mainly known for its neuroprotective properties, QUIN is known to have more neurotoxic effects (see review by Nemeth, Toldi et al. 2005). Kynurenine is able to cross the BBB a very limited degree. In the brain, 40% of kynurenine is produced locally, whereas 60% originates from the periphery (Gál and Sherman 1980). QUIN induces neurotoxic effects through its action as agonist of N-methyl-D-aspartate glutamate (NMDA) receptors, including excitotoxic neuronal cell loss and convulsions (see review by Nemeth, Toldi et al. 2005). Stimulation of extra-synaptic NMDA receptors reduces the production of BDNF, resulting in increased depressive-like behaviour (Raison and Miller 2013). It is proposed that the production and accumulation of QUIN in the CNS occurs in a variety of inflammatory neurological disorders as well as infections (Dang, Dale et al. 2000). For instance, higher QUIN levels

were observed in activated microglia in the anterior cingulate cortex of suicide victims who suffered from depression (Raison and Miller 2013). QUIN also intensifies neurotoxic effects of pro-inflammatory cytokines (see review by Maes 2008). Other neurotoxic effects caused by QUIN are the induction of hippocampal cell death, selective necrosis of granule cells, destruction of postsynaptic elements and a reduction in cerebral cholinergic circuits (el-Defrawy, Boegman et al. 1986, Garthwaite and Garthwaite 1987, see review by Maes, Yirmiya et al. 2009). Furthermore, 3HK and 3HAA have been shown to exert negative effects, such as apoptosis, neuronal damage and oxidative stress throughout the generation of reactive oxygen species (Mackay, Forrest et al. 2006). KYNA acts as a potentially neuroprotective product due to its antagonist affinity for NMDA receptors (Perkins and Stone 1982). On the other hand, increased levels of KYNA have also been associated with cognitive deficits in schizophrenia patients, which may be related to disturbed glutamatergic neurotransmission. A study showed that infusion of nanomolar concentrations of KYNA, or production in rats *in situ*, reduced extracellular glutamate in the prefrontal cortex by inhibition of $\alpha 7$ nicotinic acetylcholine receptors ($\alpha 7$ AChR) (Wu, Pereira et al. 2010). When cortisol is increased (like in case of stress), TDO is stimulated by GCs, resulting in increased kynurenine levels, the metabolism of which also occurs extra-hepatically. In addition, KMO is stimulated by pro-inflammatory cytokines. Therefore, in inflammation, kynurenine is further metabolised to 3HK (and

subsequently QUIN), and this arm of the pathway is favoured over the formation of KYNA. However, next to increased levels of QUIN levels, KYNA levels are higher than normally as well, which may help to neutralise adverse effects of QUIN (see review by Myint 2012).

Interestingly, in the brain, the increased metabolism of tryptophan may also result in a deficit in 5-HT, which, in case of inflammation, is not only metabolised by monoamine oxidase (MAO), but is also degraded by activated IDO (Pertz and Back 1988). Thus, IDO plays an important role in the regulation of 5-HT related mood states and the regulation of cellular immune responses (Stastny, Konstantinidis et al. 2003). As such, the kynurenine pathway connects the 5-HT and the inflammatory hypotheses in depression (Mackay, Forrest et al. 2009). In an inflammatory state, more kynurenine is available to be transported into the brain, and its metabolism in the brain is increased. The resulting metabolites of the kynurenine pathway directly affect the balance between neuroprotective and neurodegenerative processes in the brain (see review by Myint 2012). When microglia are activated, QUIN and 3HAA are excessively synthesised and astrocytes are not capable to metabolise the excess of QUIN, resulting in a loss of astrocytes due to the apoptotic pro-effect of 3HK (see review by Leonard and Myint 2009). With the imbalance in those metabolites, neurons are primary exposed to the neurodegenerative effects of QUIN, which together with a decreased neuronal repair, results in a variety of structural and functional abnormalities in the brain (see review by Myint 2012).

Some studies have examined monoamine levels in offspring after prenatal exposure to an infectious agent. Administration of Poly I:C during pregnancy in both rats and mice led to reduced hippocampal 5-HT levels in adult offspring (Winter, Djodari-Irani et al. 2009, Ohkawara, Katsuyama et al. 2015). Studies show contradictory results about dopamine levels in offspring after exposure to an infection during pregnancy. On the one hand, elevated dopamine and 3,4-Dihydroxyphenylacetic acid (DOPAC) were found in the lateral globus pallidus and prefrontal cortex in offspring exposed to maternal Poly I:C, but no significant change was observed in striatal regions (Winter, Djodari-Irani et al. 2009). On the other hand, another study showed increased DOPAC in the offspring striatum (Ozawa, Hashimoto et al. 2006). To date, only one study has analysed the metabolites of the kynurenine pathway in both control and offspring exposed to maternal infection reporting elevated serum QUIN and reduced KYNA and kynurenine in preadolescence rat offspring exposed to prenatal maternal Poly I:C (Zavitsanou, Lim et al. 2014).

Potentially, the kynurenine pathway may represent a key player linking various processes known to be dysregulated in psychiatric disorders. As commented above, as several enzymes intervene in the production of QUIN and KYNA, the complex interaction between the various enzymes, neurotransmitters and the production of different metabolites still needs to be elucidated.

2.2.1.4 Neurodegeneration hypothesis of depression

Recently, the “neurodegeneration hypothesis of depression” was proposed, by integrating the above mentioned concepts, the kynurenine pathway and its connection with cytokines, kynurenine metabolites, 5-HT as well as N-methyl-D-aspartate glutamate (NMDA)-glutamatergic neurotransmission (Myint and Kim 2003). In the end, various effects of cytokines can result in either neuroprotection or neurodegeneration. Generally, neurodegeneration is preceded by inflammation (see review by Camacho-Arroyo, Lopez-Griego et al. 2009). Although some studies indicate that inflammatory reactions in the CNS are usually considered to have neurotoxic effects, evidence suggests that they can also be beneficial (Hohlfeld, Kerschensteiner et al. 2007), such as in case of tissue repair and phagocytosis of apoptotic cells and debris (see review by Ramesh, MacLean et al. 2013). In an *in vivo* study, Schwartz and colleagues found that Th1 cytokines can produce neuroprotective and regenerated actions (Kipnis, Mizrahi et al. 2002) as Th2 does, but to a lesser extent. They introduced the term “protective autoimmunity” which deals with the beneficial side of immune cell filtration of the CNS. Various cytokines, generally depending on their concentration, exert both neurotrophic and neurotoxic effects (see review by Rothwell and Hopkins 1995). Pregnancy is dominated by a Th2-type response, whereas a Th-1 response can result in foetal loss (Wegmann, Lin et al. 1993, Raghupathy 1997). Lesions in CNS could lead to a systemic

immunosuppression if they result in a shift to a Th2 cytokine pattern. It is reported that after treatment with Th2 inducers, there is an elevation in neuroprotection and regeneration. Therefore, it has been proposed that to repair the CNS after injury completely, a part of the response includes a shift to Th2, although this shift may suppress the cellular immune response and could facilitate the predisposition to an infection (see review by Hendrix and Nitsch 2007).

Altogether, in depressive disorders, the overproduction of cytokines may disturb the loop of regulation within the neuro-endocrine-immune system and hence, resulting in an acute phase response, an hyperactive HPA axis and dysregulation of 5-HT turnover (see review by Corcos, Guilbaud et al. 2002).

3. Stress, stress related disorders, pregnancy and the immune system

Chronic and/or excessive stress has been related to several disorders including depression (Raison, Capuron et al. 2006). Interestingly, evidence shows that stress has an impact upon the immune system (see review by Segerstrom and Miller 2004), the effects of which vary depending on the duration of the stressor. Whereas acute stress upregulates the immune response, chronic stress suppresses it (Dhabhar and McEwen 1997). An increase of lymphocytes, NK cells and cytotoxic T cells has been observed after acute stress (Naliboff, Benton et al. 1991, Herbert, Cohen et al. 1994),

while chronic stress resulted in a decrease in their number (Herbert and Cohen 1993). In several ways, exposure to stress leads to similar changes as those caused by an infection. As shown in the previous sections, exposure to infectious agents during pregnancy produces changes in GCs, 5-HT and neurotrophins, among others. In this section, we focus on the effects of stress on these features and more specifically, on the effects of stress exposure during pregnancy, showing that prenatal maternal stress and infection may have similar consequences in the offspring. For instance, dysregulation of the HPA axis and the concomitant release of GCs can both be induced by stress (see review by Levy and Tasker 2012) and by the induction of cytokines after exposure to infectious agents (see review by Silverman, Pearce et al. 2005). Acute stress results in a transient increase in GCs that activate receptors in the pituitary and hypothalamus. Thus, decreasing the release of CRF, ACTH and GCs. In case of chronic stress, a hypersecretion of GCs is produced together with a sustained activation of the central and peripheral sympathetic nervous system as a result of the desensitisation of the central GR. Subsequently, the impaired negative feedback regulation induces a chronic hypersecretion of cortisol (Leonard 2006). Another example is the availability of 5-HT, which is also affected by hypersecretion of GCs. Acute stress provokes an increased turnover of 5-HT, which is connected with an induction of tryptophan hydroxylase activity (enzyme involved in the synthesis of 5-HT) (Davis, Stanford et al. 1995). However, chronic stress causes a reduction in 5-HT turnover via stimulation of tryptophan 2,3-dioxygenase in the liver and the consequent enhanced metabolism of tryptophan throughout kynurenine pathway (Leonard 2006).

Consequently, the 5HT_{1A} receptor is also affected by the availability of 5-HT, being increased its response in acute stress and decreased under chronic stress conditions. Under chronic stress conditions, 5HT_{1A} receptors in the hippocampus are further inhibited by GCs (Chaouloff 1995, Meijer and de Kloet 1998).

In order to study the effects of prenatal stress in the offspring, several animal studies have been conducted exposing the pregnant dam to stress and, subsequently, examining the effects in the offspring. As an example, offspring exposed to prenatal stress showed an increased CRF release and levels in the amygdala (Cratty, Ward et al. 1995). In another study, Huang and colleagues subjected pregnant dams to stress and they also found increased corticosterone and CRF levels in the offspring. Moreover, 5-HT levels were increased in the hippocampus and hypothalamus of foetuses exposed to prenatal stress, whereas only hippocampal 5-HIAA levels were lower compared to control foetuses. In addition, 5-HT_{1A} receptor levels were significantly reduced in the hippocampus and levels of the serotonin transporter (SERT) were lower in both the hippocampus and hypothalamus of foetuses exposed to prenatal stress (Huang, Xu et al. 2012). Regarding neurotrophic factors, for example, reduced brain BDNF content was observed just after birth in prenatally stressed offspring (Van den Hove, Steinbusch et al. 2006). Changes in cytokines in the offspring have also been investigated after prenatal stress showing an increased expression of IL-1 β and TNF- α in the hippocampus and increased percentage of reactive microglia cells in CA1 compared to non-stressed offspring (Diz-Chaves, Astiz et al. 2013). However, evidence is lacking when analyzing the involvement

of the kynurenine pathway in prenatally stressed offspring. Only in 1990, a study reported increased brain tryptophan levels in foetus exposed to prenatal stress (Peters 1990).

To sum up, exposure to stress or infection, in particular during pregnancy, may lead to similar changes in the immune system and in GCs, 5-HT and neurotrophines, among others, although the exact functional link between prenatal maternal infection and stress remains to be elucidated.

Figure 1. Integrated view of the potential mechanisms underlying the relationship between prenatal maternal infection and stress on adult psychopathology, particularly depression. The diagram shows that exposure to an infection or stress during the pregnancy results in increased immune activity. This response leads to an increase in pro-inflammatory cytokines, activation of the kynurenine pathway and the hypothalamic pituitary adrenal axis, concomitant with an increase in glucocorticoids levels in both the mother and the foetus. As a consequence, a decrease in serotonin is being induced. Glucocorticoids and cytokines can cross the blood brain barrier, whereas the activation of the kynurenine pathway in the foetus may also affect neurodevelopment in the offspring. Adult offspring prenatally exposed to infection or stress showed reduced serotonin and disturbed behaviour, which together with epigenetic changes and enhanced kynurenine pathway may lead to the development of depression. Abbreviations: IA, immune activation; HPA, hypothalamic pituitary adrenal; GCs, glucocorticoids; 5-HT, serotonin; KP, kynurenine pathway.

4. Discussion

4.1 Gender differences (offspring psychopathology in response to prenatal maternal infection)

Mental disorders affect men and women differently but the exact mechanisms that cause this distinction remain unknown. For instance, the prevalence of depression in women has been reported to be 1.7 to 2.7 times higher than in men (see review by MacQueen and Chokka 2004). Most likely, a combination of various factors, such as hormonal fluctuations to which women are subjected -which are known to influence neurotransmitters- accounts for this gender variation (see review by Vigod and Stewart 2009). Furthermore, the modulatory function of those hormones and their interaction with other hormonal systems such as the HPA axis might increase the vulnerability to depression predominantly in women (see review by Steiner, Dunn et al. 2003). Interestingly, placental 11 β HSD2 activity has been reported to be reduced in case of females foetuses after inflammation, whereas in male foetuses an increase in 11 β HSD2 activity has been observed (Murphy, Gibson et al. 2003, see review by Howerton and Bale 2012), thereby particularly exposing female foetuses at risk to elevated GC exposure. Even during early CNS development, the interaction between neuroendocrine and neuroimmune pathways mediates the establishment of sex differences (Bale 2009). Immune cells express receptors for sex hormones and, as such, are also modulated by gonadal hormones (Klein, Roberts et al. 2010). It is already known that the immune response differs between males and females (see review by Fish

2008). For instance, women show an increased immune response after exposure to viral infections (Klein and Huber 2010). Specifically, the Influenza virus has been shown to provoke an excessive pro-inflammatory cytokine response (de Jong, Simmons et al. 2006), which was more pronounced in females compared to males (see review by Klein, Hodgson et al. 2012). Over the last years, there has been mounting evidence for effects in the offspring after prenatal maternal infection. However, only a limited number of studies have been conducted to analyse sex differences after prenatal infection. Lante et al. showed that prenatal LPS exposure in rats led to oxidative stress only in the hippocampus of male fetuses (Lanté, Meunier et al. 2007). Another group examined the behavioural effects after prenatal exposure to LPS. Male offspring showed stronger anxiety-driven reactions and impaired spatial learning and memory (Chlodzinska, Gajerska et al. 2011). Moreover, male offspring exposed prenatally to LPS showed elevated numbers of macrophage and infiltrating cells and incremented loss of hippocampal volume (Dada, Rosenzweig et al. 2014). However, another study using prenatal exposure to LPS reported altered behaviour especially in females, such as sensorimotor impairment (Wang, Meng et al. 2010). In relation to autism, a recent study showed that male offspring prenatally exposed to both LPS and Poly I:C showed increased repetitive behaviour. In addition, decreased motor activity was observed only in male offspring from Poly I:C challenged dams (Xuan and Hampson 2014). A study focussing on schizophrenia-related phenotypes showed different effects after prenatal infection between male and female as well. Altered PPI was

reported in both pubertal and adult males, but only in pubertal females after LPS injection to the pregnant rat. In addition, males showed impaired recognition memory for objects to a greater extent. Finally, in male offspring prenatally exposed to LPS, a more pronounced reduction of myelination in cortical and limbic brain regions and decreased parvalbumin-expressing cells in the medial prefrontal cortex, hippocampus and entorhinal cortex has been observed compared to females (Wischhof, Irrsack et al. 2015).

Altogether, it is important to consider sex differences when assessing a subject's response to neuroendocrine or immune challenges early in life, such as prenatal infection or stress, and how such perturbations might then program future responses to similar challenges (Bale 2009).

4.2 Epigenetics

Recently, the effects of epigenetic marks in the nervous system have been raised the attention of researchers. Epigenetic modifications are heritable changes in gene expression that do not involve alterations of the DNA sequence. The most-studied epigenetic mechanisms to date are DNA methylation, histone modifications and noncoding RNA expression. Diverse functions in the nervous system are under the influence of epigenetic mechanisms, such as neuronal-glial differentiation, modulation of neural plasticity and behaviour, adult neurogenesis, memory and cognition (see review by Ravi and Kannan 2013). Furthermore, prenatal infection is reported to produce epigenetic modifications of CNS genes, resulting in altered structure or function of

specific neuronal pathways (see review by Boksa 2010). It is already known that in response to a microbial exposure, cells exert modifications in the transcriptional program of specific genes in order to induce a competent response. However, pathogens have been shown to interrupt this response to ensure an adequate environment for their survival (Hamon, Batsché et al. 2007). Thus, the target host genes for epigenetic control are genes involved in cell cycle progression, survival, inflammation and immunity (Al Akeel 2013). The activation or silencing of genes is produced by transcription factors as well as epigenetic modulators (see review by Bierne, Hamon et al. 2012), and both of them have been shown to be altered by pathogens. More specifically, histone modifications are the main epigenetic processes affected by bacteria. One example is the increase in histone acetylation and phosphorylation, specifically at the promoter of IL-8 in dendritic cells, after LPS exposure (Saccani, Pantano et al. 2002). Recent evidence suggest that the regulation of histone acetylation, by directly modifying acetylation or through impact upon histone deacetylase (HDAC) activity, on host defense genes may be a common characteristic of bacterial infections (see review by Garcia-Garcia, Barat et al. 2009, Paschos and Allday 2010). For instance, inhibition of histone 3 acetylation and phosphorylation impairs the host innate immune response. But also changes in the expression of HDACs have been shown, resulting in silencing of host defense genes (see review by Bierne, Hamon et al. 2012). In addition, DNA methyltransferase (DNMT) expression has been also found to be increased (Al Akeel 2013). Several viruses have been shown to modulate

epigenetic mechanisms. For instance, increased expression of DNMTs is found in hepatitis B virus-infected cells (Vivekanandan, Daniel et al. 2010), whereas DNA hypermethylation has been associated with the presence of human papillomavirus (Katargin, Pavlova et al. 2009). But also histones are modulated by virus pathogens, such as the adenovirus, which reduces acetylation of histone 3 (Horwitz, Zhang et al. 2008). With respect to pregnancy infection, only a limited number of studies have been conducted to analyze the epigenetic changes in the offspring exposed to prenatal infection. One example shows reduced DNA methylation in the hypothalamus of adolescent murine offspring, after prenatal Poly I:C acid exposure (Basil, Li et al. 2014). In another study, exposing pregnant mice to Poly I:C was associated with global hypoacetylation of histone H3 and histone H4 in the cortex of juvenile offspring (Tang, Jia et al. 2013).

All in all, infections can modulate epigenetic processes in many ways (see review by Bierne, Hamon et al. 2012). Epigenetic reprogramming is being employed by cells as an adaptive mechanism to ensure a favorable metabolic status after exposure to physiological substances or infectious agents (Waly, Hornig et al. 2012). As mentioned above, epigenetic regulation plays an important role in the development and differentiation of the nervous system that may serve to explain the molecular basis of the CNS response in case of injury after exposure to infection. Clearly, the magnitude and exact involvement of epigenetic modifications in mediating changes in brain function and behaviour in response to prenatal maternal infection remains to be disclosed.

4.3 Treatment options

Many types of treatments have been developed in order to alleviate the symptoms of depression. However, to date, the overall efficacy of current treatment strategies is limited and still a considerable number of patients show recurrence (see review by Burcusa and Iacono 2007). Examples of common treatments applied are psychotherapy, antidepressants and electroconvulsive therapy (ECT), among others. Currently, antidepressants, such as selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs) and monoamine oxidase (MAO) inhibitors, in combination with psychotherapy are the preferred choice. However, as a consequence of their low efficacy in some patients (Nelson 1998, Fournier, DeRubeis et al. 2010), new strategies have been investigated, such as HDAC inhibitors. HDAC inhibitors are involved in transcriptional regulation of cytokines, in immunologic signaling pathways, and inflammatory responses (see review by Qureshi and Mehler 2011). On the one hand, the commonly used HDAC inhibitor valproic acid (VPA), is found to dedifferentiate committed oligodendrocytes precursors to multipotent neural progenitors, thus inducing neuronal differentiation (Liu, Han et al. 2007). On the other hand, HDAC inhibitors are known to produce adverse side effects. Thus, their use in psychiatric disorders is still under discussion.

4.4 Summary and outlook

This review addressed the possible mechanisms through immune activation during pregnancy may lead to development of psychiatric disorders in the offspring. Infections by bacteria or other virus during pregnancy increase the risk for the development of mental disorders in the offspring. Among others, cytokines, associated with a pathogenic immune response are thought to impact upon foetal brain development. It is still unknown, however, how exactly viral or bacterial exposure leads to pathology in the CNS (Zuckerman and Weiner 2005). Many experiments in mice and rats have provided evidence about abnormalities in brain development and structure in offspring after prenatal exposure to pathogens, such as synthetic double-stranded RNA Poly I:C, which is reported to provoke a decrease in hippocampal function in adulthood (Zuckerman, Rehavi et al. 2003). Moreover, epigenetic processes may represent an important player in mediating the molecular changes that may contribute to the development of psychiatric disorders.

Further studies should be done to understand how prenatal environmental insults can affect neuropathological and psychopathological brain development of offspring and to develop preventive strategies. A detailed analysis of the kynurenine pathway (metabolites and enzymes) could be very helpful in order to improve diagnose and treatment of psychiatric disorders.

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Chapter 3

Effects of subcutaneous LPS injection on gestational length and intra-uterine and neonatal mortality in mice

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Abstract

Infection during pregnancy can predispose offspring to develop various psychiatric disorders such as depression in later life. In order to investigate potential mechanisms underlying these associations, animal models of maternal infection have been employed. As such, lipopolysaccharide (LPS) has been commonly used to mimic a bacterial infection in pregnant mice. The original aim of our study was to investigate the effects of different doses of subcutaneous lipopolysaccharide (LPS) administration on affective behaviour in adult mouse offspring. In the present paper, however, we report that subcutaneous LPS administration has a profound impact upon gestational length, litter size and perinatal mortality in the offspring, even at a relatively low dose.

Pregnant mice were randomly divided into 3 groups, receiving either a high (2 mg/kg) or low (0.5 mg/kg) dose of LPS or phosphate buffered saline (PBS) by means of subcutaneous injection. Subsequently, the effects on gestational length, litter size and perinatal mortality in the offspring were assessed. After subcutaneous injection with a high dose of LPS, we observed a significant decrease in gestational length and an increase in neonatal mortality. When the low dose administered, a tendency towards reduced litter size was observed, most likely reflecting increased intra-uterine mortality in response to prenatal maternal LPS exposure.

We show that subcutaneous LPS administration of 2 mg/kg to pregnant mice in the last phase of gestation should be avoided because of high offspring mortality rates, whereas the subcutaneous injection of 0.5 mg/kg of LPS seems to result in the reabsorption of foetuses.

1. Introduction

Infection during pregnancy has been reported to increase the susceptibility of the offspring to develop psychopathology later in life (Patterson 2002, Meyer, Yee et al. 2007). For instance, increased risks of schizophrenia, bipolar disorder and autism have been reported in offspring of women infected with viruses or bacteria during pregnancy (Brown 2006, Atladottir, Thorsen et al. 2010, Parboosing, Bao et al. 2013). In order to investigate potential mechanisms underlying these associations, animal models of maternal infection have been employed. As such, lipopolysaccharide (LPS) has been commonly used to mimic a bacterial infection in pregnant mice. Through the toll-like receptor 4 (TLR4), LPS increases the production of pro-inflammatory cytokines, which are known to interfere with the development of the central nervous system (Boks 2010). In the fetal brain, cytokines are known to regulate the function and development of neurons, being involved in neuroprotective and neurodegenerative processes (Licinio 1997). Different effects of LPS in rodents have been reported, such as modulation of the hypothalamus-pituitary-adrenal (HPA) axis, resulting in increased corticosterone levels (Grinevich, Ma et al. 2001). After LPS exposure during gestation, several behavioural changes have been observed in the offspring in adulthood, e.g. impairments in spatial learning and memory (Golan, Lev et al. 2005, Lante, Meunier et al. 2008, Hao, Hao et al. 2010).

Intraperitoneal (i.p.) injection of LPS to pregnant mothers is known to result in fetal death depending on the dose administered (Renaud, Cotechini et al. 2011). In addition to the dose injected, the time point of LPS injection plays an important role regarding changes in protein levels of cytokines in the

brains of foetuses (Chlodzinska, Gajerska et al. 2011). Noteworthy, the great majority of prenatal LPS studies have used i.p. injections in pregnant mice. (Buhimschi, Buhimschi et al. 2003, Xu, Chen et al. 2006, Salminen, Paananen et al. 2008, Chlodzinska, Gajerska et al. 2011, Renaud, Cotechini et al. 2011). Considering the fact that i.p. injections have a failure rate in the order of 10–20% (Gaines Das and North 2007), subcutaneous (s.c.) injections may be favored – particularly when dealing with pregnant animals, where misplacement of the injection can cause severe damage to both the mother and foetus. However, little is known about potentially adverse effects of s.c. LPS injections in pregnant animals. Whereas originally, the aim of our study was to investigate the effects of different doses of s.c. LPS administration on affective behaviour in adult offspring, in the present paper, we report that s.c. LPS administration has a profound impact upon gestational length, litter size and perinatal mortality in the offspring, even at a relatively low dose.

2. Material and methods

2.1.1 Animals

C57BL/6JRccHsd mice were obtained from Harlan Laboratories (Eystrup, Germany). Mice were housed 4 per cage in single-sex groups in individually ventilated cages (IVC) with cage sizes 480 x 375 x 210 mm (depth x width x high) under specified pathogen free (SPF) conditions and maintained on a 12/12h light-dark cycle (light on at 12am) and temperature-controlled environment (relativity humidity: 55% $\pm$ 5%; temperature: 22°C $\pm$ 2°C; room air exchange rate: 15). Mice were s.c.

injected with a transponder for identification purposes. Breeding food (Ssniff, Germany) and water were allowed ad libitum. Cages were changed every week. Experiments were conducted in accordance with permissions from the government and the veterinarian administration of Oberbayern (AZ: 55.2-1-54-2531-61-10). After arriving at the local animal facilities, the mice were allowed to habituate to their new environment for 3 weeks. During this period, mice were chipped to facilitate their identification. For mating, females were housed together with a male mouse for 3 days (1 male and 1 female per cage). Afterwards, females were housed individually. Three weeks after delivery, offspring were weaned and separated by sex (4 mice/cage). Offspring were chipped when they were 6 weeks old.

2.1.2 Mating and induction of prenatal infection

Approximately 8 weeks after birth, male and female mice were coupled again for 3 days. At GD17, pregnancy was confirmed by visual inspection of the dam in combination with weight gain analysis (increase between day of mating and the day of the injection). Pregnant mice were randomly divided into 3 groups (high dose of LPS [n=21], a low dose of LPS [n=16] and phosphate buffered saline [PBS; n=20]). Dams were s.c. injected with 0.5 or 2 mg/kg LPS (from *Salmonella enterica* serotype enteritidis, Sigma Aldrich (Germany)) or an equivalent volume of PBS. Following injection, mice were observed daily until delivery. Postnatal mortality was calculated by comparing the number of born pups found dead and the total number of pups counted during the observation period. Of note,

when assessing postnatal mortality, 4 animals were excluded in the group treated with a low dose of LPS, as these 4 animals were confirmed to be pregnant at GD17, but never delivered.

2.2 Statistical analysis

To define statistically significant differences in gestational length, litter size and mortality, data were first subjected to analysis of variance (ANOVA) with IBM SPSS Statistics 20 (SPSS Inc., USA). Significant overall effects were subsequently examined in more detail using post-hoc Bonferroni tests. The level of significance was set at $p < 0.05$ in all cases.

3. Results

Overall, significant effects of LPS administration were found for gestational length ($F_{2,54}=26.366$; $p<0.001$) and neonatal mortality ($F_{2,50}=93.997$; $p<0.001$), while a trend towards a significant effect on litter size ($F_{2,54}=2.700$; $p=0.076$) was observed (see Figure 1). Already one day after injection, some dams treated with the high dose of LPS started to deliver. This led to a significant decrease in gestational length in mothers treated with the high dose of LPS when compared to the control group ($p<0.001$), while no difference in gestational length was observed between dams injected with the low dose of LPS and control dams ($p=1$). LPS mothers given the high dose showed a reduction in gestational length compared to those injected with a low dose ($p<0.001$). In addition, litter size tended to be reduced in dams injected with a low dose of LPS when compared to controls, although this difference did not reach statistical significance ($p=0.095$). Further, no

76

significant differences were observed between the high dose of LPS and controls ($p=1$); and between the two doses of LPS ($p=0.211$). A remarkable effect was observed regarding neonatal mortality. After injection with the high dose of LPS, almost all pups died within a few days after birth ($>95\%$), whereas mortality in control offspring was almost negligible (1.5% , $p<0.001$). A mortality rate of 16.67% was detected following administration of the low dose of LPS, but this effect did not reach significance when compared to controls ($p=0.233$). When comparing both doses of LPS, a significant increase in mortality was observed in pups from mothers injected with a high dose of LPS ($p<0.001$).

Figure 1. Overall, significant LPS effects were found on gestational length ($F_{2,54}=26.366$; $p<0.001$) and mortality ($F_{2,50}=93.997$; $p<0.001$), while a trend towards a significant effect on litter size ($F_{2,54}=2.700$; $p=0.076$) was observed.

a: More specifically, a reduced gestational length was observed after subcutaneous administration of a high dose of LPS when compared to control ($p<0.001$). A similar effect was found when analyzing both doses of LPS ($p<0.001$).

b: Litter size tended to be reduced after treatment with a low dose of LPS ($p=0.095$) when compared to controls.

c: after subcutaneous injection with the high dose of LPS, neonatal mortality was increased when compared to both the low dose of LPS ($p<0.001$) and controls ($p<0.001$). Bars represent means + S.E. # $p<0.1$, ## $p<0.001$ (ANOVA), * $p<0.1$, ** $p<0.001$ (Bonferroni).

4. Discussion

In the present paper, we report that s.c. LPS administration in the mouse has a profound impact upon gestational length, litter size and perinatal mortality in the offspring, even at a relatively low dose. More specifically, our study shows that s.c. administration of LPS at a dose of 2 mg/kg to pregnant mice resulted in a significant reduction in gestational length and a high *neonatal* mortality rate, whereas the s.c. injection with 0.5 mg/kg LPS seemed to be associated with increased *prenatal* mortality, reflecting reabsorption of foetuses.

4.1 Route of administration

Whereas other publications reported similar effects after prenatal LPS administration, the exact effects seem to largely depend on the dose, route of administration and gestational day of exposure or on the combination of these factors (Mijovic, Zakar et al. 2002, Nakamura, Mori et al. 2003). Most of the studies used i.p. administration of LPS (e.g. (Xu, Chen et al. 2006, Salminen, Paananen et al. 2008)), while little is known about other ways of administration such as intrauterine or intravenous injections (Kohmura, Kirikae et al. 2000, Wang, Hagberg et al. 2007). Importantly, between 10 and 20% of regular i.p. injections are not placed in the peritoneal cavity, but in the intestine or stomach and this frequency could not be minimized after modifications of the procedure (Steward, Ornellas et al. 1968, Miner, Koehler et al. 1969, Gaines Das and North 2007). In fact, in case of injecting pregnant dams, such adverse effects may be expected to be even worse, including direct harm to the foetus.

In addition, other disadvantages such as splenic trauma, serosal hemorrhage and contamination are reported (Cho, Lee et al. 2011). Hence, s.c. injections may represent more a more suitable route of administration when dealing with pregnant mice.

4.2 Dose dependent effect on prenatal and neonatal mortality

As mentioned above, the dose of LPS and the timing of the injection are considered to be critical factors that may impact upon fetal death. However, to date, no report has indicated which dose of s.c. LPS is sufficient to trigger a response in the mother and foetus without causing death. A limited number of articles have been published studying the effects of zinc supplementation and teratogenicity after prenatal exposure to LPS (Carey, Berbee et al. 2003, Chua, Rofe et al. 2006, Chua, Cowley et al. 2012). However, no evidence was reported on effects on intra-uterine or neonatal mortality, litter size or gestational length. In the present study, litter size tended to be reduced when using a low dose of LPS. In fact, 4 females within this group were confirmed to be pregnant at the time of LPS injection (GD17), but never delivered, pointing at reabsorption of foetuses. Interestingly, it has been reported that prenatal maternal i.p. administration of high doses of LPS causes fetal resorption, whereas exposure to low doses resulted in normal delivery (Chlodzinska, Gajerska et al. 2011). Contra-intuitively, we only observed a reduction in litter size after prenatal administration of a low dose, but not after using a high dose of LPS. This lack of effect when using the high dose could be explained by the fact that the high dose of LPS induced an abrupt

premature delivery, concomitant with a massive increase in *neonatal* – instead of *prenatal* – mortality, allowing no opportunity for intrauterine absorption. Although it was expected that some pups would die after exposure to LPS, nonetheless, the high rate of neonatal mortality (>95%) observed after administration of a high dose of LPS compared to the low dose (16.67%) was remarkable.

4.3 Concluding remarks

Evidently, it would be useful, to find out more about the exact effects of prenatal LPS administration in pups when using different doses, routes and time points of administration in order to choose the suitable exposure for future experiments. Although most problems can be expected when using i.p. injections, this route of administration is still mostly employed in mouse models. Therefore, we investigated the effects of different doses of LPS using s.c. injections. In our study, using s.c. LPS injections, we could avoid the problem of misplacing the agent or directly harming the foetus when injecting the pregnant dams. Altogether, we conclude that s.c. LPS administration in the mouse has a profound impact upon gestational length, litter size and perinatal mortality in the offspring, even at a relatively low dose. Future work should elucidate to which extent lower doses of LPS induce short- and/or long-term behavioural changes in the offspring and which exact molecular mechanisms are involved.

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Chapter 4

Quinolinic acid-immunoreactivity in the naïve mouse brain

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Abstract

Quinolinic acid (QUIN) has been suggested to be involved in infections, inflammatory neurological disorders and in the development of psychiatric disorders. In this view, several studies have been performed to investigate QUIN localization in the brain and its neurotoxic effects. However, evidence is lacking regarding QUIN in healthy, control conditions. The aim of this study was to investigate the region-specific distribution and pattern of QUIN expression in the naïve mouse brain. In addition, possible sex differences in QUIN-immunoreactivity and its link with affect-related behavioural observations were assessed.

For this purpose, naïve mice were subjected to the forced swim test (FST) and 20 minutes open field (OF) testing to measure affect-related behaviour. Afterwards, brains were assessed for QUIN-immunoreactivity. QUIN-immunoreactivity was particularly observed in the cingulate cortex (CC), highlighting clearly delineated cells, and the thalamic reticular nucleus (TRN), showing a more diffuse staining pattern. Subsequently, QUIN-positive cells in the CC were counted, while QUIN-immunoreactivity in the TRN was examined using gray value measurements. No significant differences between sexes were observed for the number of QUIN-positive cells in the CC, neither in levels of QUIN-immunoreactivity in the TRN. A direct correlation was found between QUIN-positive cells in the CC and QUIN-immunoreactivity in the TRN. Moreover, in male mice, a very strong correlation ($r_{sp}=0.943$; $p<0.01$) between QUIN-immunoreactivity at the level of the TRN and motor activity in the OF was observed. Thus, our results

suggest that QUIN –detected in the CC and the TRN- may play an important role in regulating motor activity in normal conditions.

1. Introduction

Quinolinic acid (QUIN) is an endogenous N-methyl-D-aspartate (NMDA) glutamate receptor agonist produced by the kynurenine pathway, particularly in the liver and the brain. The kynurenine pathway, which has been suggested to be implicated in the development of psychiatric disorders (Asp, Johansson et al. 2011, Myint 2012), involves the catabolism of tryptophan to kynurenine, which in turn can be further metabolised into the intermediates kynurenic acid (KYNA) or QUIN. Alterations in tryptophan, kynurenine, KYNA and QUIN have been reported in patients suffering from depression, schizophrenia and Alzheimer's disease, among others (Nilsson, Linderholm et al. , Németh, Toldi et al. 2006, Myint, Kim et al. 2007, Gulaj, Pawlak et al. 2010). In depressive patients, increased cerebrospinal fluid (CSF) levels of QUIN have been observed (Raison, Dantzer et al. 2010), whereas another recent study showed decreased QUIN-immunoreactivity in the hippocampus of patients suffering from depression (Busse, Busse et al. 2014).

Evidence suggests that the production and accumulation of QUIN in the central nervous system (CNS) is implicated in several inflammatory neurological disorders and infections (Dang, Dale et al. 2000). It is well-known that during inflammation, cytokines are produced which mediate a subset of distinct inflammatory reactions (Kronfol and Remick 2000, Zhang and An 2007). Already in 1992, a study reported increased QUIN in lumbar CSF and post-mortem brain tissue of patients with inflammatory diseases, but not in CSF of patients with non-inflammatory neurological disease (Heyes, Saito et al. 1992). Moreover, in depression associated with a pro-

90

inflammatory status, QUIN-immunoreactivity was found to be increased in the anterior midcingulate cortex (aMCC), the subgenual anterior cingulate cortex (sACC) and the cortical grey matter (Steiner, Walter et al. 2011). QUIN has been reported to aggravate the negative consequences of pro-inflammatory cytokines (Maes 2008) and to exert various neurotoxic effects, inducing neuronal cell loss and convulsions (Nemeth, Toldi et al. 2005). QUIN has also been linked to the induction of hippocampal cell death, selective necrosis of granule cells, destruction of postsynaptic elements and a reduction in cerebral cholinergic circuits (Maes, Yirmiya et al. 2009).

In the CNS, QUIN is produced by activated microglia and macrophages. Different studies with discordant results have been conducted using several animal models and human tissues to determine the cellular localisation of QUIN in the brain. In brain tissue from naïve control rats, no QUIN-immunoreactivity was observed (Moffett, Espey et al. 1993). However, different types of QUIN-positive cells, such as microglia, macrophage and ameboid-like cells were seen in the subiculum, dorsal hippocampus (CA1) and in the stratum pyramidale of the whole CA1 area in a gerbil model of ischemia (Baratte, Molinari et al. 1998). In another study, high QUIN-immunoreactivity was observed only after focal injury of the neocortex in adult rats and was limited to those microglia directly within the lesion tract. In addition, QUIN-immunoreactivity was also seen in reactive cultured microglia and also in microglia of primary cultures of human mixed brain cells after IFN- γ stimulation (Guillemin, Smythe et al. 2005, Chung, Leung et al. 2009).

Despite the fact that research within this field has rapidly expanded in the context of pathological conditions, the normal, physiological role of QUIN is less clear. To date, no report showed the localization of QUIN and the pattern of its staining in the brain of naïve mice. Therefore, we analyzed QUIN-immunoreactivity in the naïve mouse brain. Our first objective was to examine the distribution and localization of QUIN-immunoreactivity. Second, we evaluated whether QUIN-immunoreactivity differs between sexes. Third and last, we investigated the possible association between QUIN-immunoreactivity and a subset of affect-related behavioural phenotypes.

2. Material and methods

2.1 Animals

C57BL/6JRccHsd mice were obtained from Harlan Laboratories (Eystrup, Germany). Mice were housed in single-sex groups of 4 mice per cage in individually ventilated cages (IVC 480 x 375 x 210 mm [depth x width x high]). Mice were kept under specific pathogen-free (SPF) conditions and maintained on a 12/12h light-dark cycle (light on at 12am) and temperature-controlled environment (relative humidity 55% $\pm$ 5%; temperature: 22°C $\pm$ 2°C, room air exchange rate 15). Cages were changed every week. The habituation period lasted 3 weeks once the mice arrived to the local animal facilities. During this period, mice were subcutaneously injected with a transponder to facilitate identification. Breeding food (Ssniff, Germany) and water were allowed ad libitum. Experiments were performed according to the permissions from the

government and the veterinarian administration of Oberbayern (AZ: 55.2-1-54-2531-61-10).

2.2 Behavioural tests

2.2.1 Forced swim test

At 12 weeks of age, mice were tested in the forced swim test (FST). This test consists of 4 vertical glass cylinders (height 57,5cm x diameter 20) filled to a depth of 50 cm with tap water at 22°C. Cylinders were situated in front of a frosted glass screen and were illuminated by 4 infrared floodlights (Videor, Germany) behind the screen. Cylinders were separated by opaque partitions so that mice could not see each other. One animal was placed in each cylinder and tested for 6 minutes, between 12 p.m and 5 p.m. After each test, water was changed and mice were dried and put back into their home cages. A wall-mounted camera (type EQ-150, EverFocus, Germany) connected to a converter (ADVC-55 Advanced DV Converter, canopus GmbH, Germany) was used to record the test. Biobserve Forced Swim Test Version 1.5 software (BIOobserve, Germany) was employed to analyse the following behaviours: 1) floating (absence of any movement or minor movements needed to keep the head above the water), 2) swimming (movements of the limbs associated with swimming and less severe than those associated with climbing) and 3) struggling (large movements associated with attempts to escape the swim tank).

2.2.2 Open Field Test

One week after the FST, mice were tested in the open field test (OF), consisting of a white PVC box (50 x 50 x 50 cm; Kämpel Kunststoffverarbeitungswerke GmbH, Germany). Two infrared floodlights (type WFL-II/LED30, Videor, Germany), mounted on tripods (type 3150, Cullmann, Germany) were situated on two sides of the OF area (containing 4 boxes). One animal was placed in each box and was allowed to explore the arena for 20 minutes. Testing was performed between 12 p.m and 5 p.m. Boxes were cleaned after each measurement with water and alcohol and mice were returned to their cages. Behaviour was recorded with a camera (type AU-CB600, VIDO, Austria) connected to a converter (ADVC-55 Advanced DV Converter, canopus GmbH, Germany). The video-tracking software Viewer Version 3 (BIOobserve, Germany) was used to score the time spent in each zone (s), track length (cm) and zone crossings (counts) in the whole box. Each box was divided as follows: the centre (34,6 x 34,6 cm; W x D), 4 walls (each: 7 x 34,6 cm; W x D) and 4 corners (each: 7 x 7 cm; W x D).

2.3 Perfusion and extraction of organs

Thirteen week old mice were perfused 1 month after OF testing. Male [n=6] and female [n=7] mice were anesthetized with a single intraperitoneal injection of ketamin (Pfizer, Germany) and xylacin (Bayer, Germany). Subsequently, they were perfused with a heparinized saline solution followed by 4% paraformaldehyde (PFA) and 15% saturated picric acid. Brains were dissected and postfixed in 4% PFA overnight

before being transferred to PBS-containing sodium azide (1%) and stored at 4°C.

2.4 Immunohistochemistry

Thirty μm coronal brain sections of the brains were cut using a vibratome (Leica VT1200 S, Germany). Free-floating slices were rinsed for 3 times (Tris-buffered Saline with 0.3% Triton X-100 (TBST) / Tris-buffered Saline (TBS) / TBST; each for 10 min) and pre-incubated with 1% H_2O_2 in TBS to block endogenous peroxidase activity for 30 minutes at room temperature (RT). Tissue sections were washed again as mentioned above and incubated overnight at 4°C with the primary polyclonal rabbit anti-QUIN antibody (1:1500 in TBS-T, apDia, Turnout, Belgium) (Gos, Myint et al. 2014). Next, sections were washed and incubated with biotinylated donkey anti-rabbit (1:400 in TBST-T, Jackson Laboratories, US) for 90 minutes at RT. Sections were washed again and tissue was further incubated with avidin-biotin complex (ABC) (1:400 in TBS-T, Vector Immunolabs) for 90 minutes at RT. After incubation, slices were washed twice with TBS for 10 minutes and once with 0.1 M acetate buffer (pH 6.0) for 15 minutes. Subsequently, tissue was incubated for 20 minutes at RT with glucose oxidase and DAB, and washed twice for 15 minutes with 0.1M acetate buffer (pH 6.0). Finally, brain sections were mounted on coated microscope slides, dehydrated using an ethanol series, dried overnight, and coverslipped using Pertex® (HistolabProducts ab, Göteborg, Sweden).

2.5 Microscopy

Brain sections of 13 animals (6 male, 7 female) were analyzed. For cell quantification in the cingulate cortex (CC) we applied the Eclipse E400 microscope from Nikon, while for gray value analysis in the thalamic reticular nucleus (TRN) we used an AX70 microscope (Olympus, analySIS Imaging System) coupled to a digital camera (f-view, Olympus). QUIN-immunoreactivity in the TRN was detected from bregma level -0.82 mm until bregma level -1.58mm (3-4 sections). These sections were photographed at a 10x and 40x magnification and gray values were analyzed with the ImageJ software. In addition, background correction was performed by subtracting the background signal from the gray values reflecting immunoreactivity. For quantitative studies QUIN-immunoreactive cells in the CC were counted at 20x magnification. The counting was performed from bregma level 0.50 mm, where QUIN-immunoreactive cells appeared, through all the sections until bregma level -1.94mm (9 sections).

2.6 Statistical analysis

The number of QUIN-positive cells and the levels of QUIN-immunoreactivity and behavioural measurements were first subjected to non-parametric Kruskal-Wallis tests with IBM SPSS Statistics version 20 (SPSS Inc., USA). In addition, possible correlations between the number of QUIN-positive cells within the CC and the levels of QUIN-immunoreactivity in the TRN as well their correlation to behavioural measures in the FST and OF were analyzed using Spearman correlation

analysis. The level of significance was set at $p < 0.05$ in all cases. Graphs were designed with GraphPad Prism 5.

3. Results

3.1 Localization of QUIN-immunoreactivity

Low levels of QUIN-immunoreactivity were seen throughout the brain, whereas high levels of QUIN-immunoreactivity were observed specifically in two brain regions, i.e. the area ranging from the CC to the retrosplenial cortex (RSC), as well as in the TRN. To note, as the RSC is considered equivalent to the posterior CC in rodents (Vogt, Vogt et al. 2004, Vann, Aggleton et al. 2009), we will use the term CC to cover cells observed in both the CC and the RSC. QUIN-immunoreactivity in the CC clearly delineated individual cells (see Figure 1). On the other hand, QUIN-immunoreactivity in the TRN was much more diffuse, resembling a fibrillary staining pattern (see Figure 2).

Figure 1. QUIN-positive cells in the cingulate cortex. A, B) Low magnification overview of the brain showing QUIN-positive cells (2x, 4x). C, D) High magnification views of the CC (10x, 20x).

Figure 2. QUIN-immunoreactivity in the thalamic reticular nucleus. A, B) Low magnification overview of the brain showing QUIN-immunoreactivity in the TRN (2x, 4x). C, D) High magnification views of the TRN (10x, 40x).

3.2 Behavioral observations

Sex differences were found in the distance moved and number of zone crossings both during the complete 20 minutes and first 5 minutes exposure to the OF, indicative of increase activity in females compared to males (all $p < 0.05$).

3.3 QUIN-immunoreactivity in the CC and TRN; correlations to behavioural variation

Statistical analysis revealed no significant difference between sexes in the number of QUIN- positive cells within the CC ($p > 0.05$, data not shown). Regarding the TRN, no significant sex effect was found in levels of QUIN-immunoreactivity ($p > 0.05$, data not shown). Spearman correlation analysis showed a statistically significant positive correlation between the number of QUIN-positive cells in the CC and QUIN-immunoreactivity in the TRN, $r_{sp} = 0.569$ (see Table 1 and Figure 3). In addition, in males, we found a highly significant and strong positive correlation between both track length ($r_{sp} = 0.943$) and zone crossings ($r_{sp} = 0.886$) during 20 minutes' OF exposure and QUIN-immunoreactivity in the TRN (see Table 1 and Figure 3).

Table 1. Spearman correlation coefficient between QUIN-positive cells in the cingulate cortex (CC), QUIN-immunoreactivity in the thalamic reticular nucleus (TRN) and several behavioural measures; * $p < 0.05$; ** $p < 0.01$

	QUIN- immunoreactivity in the TRN	QUIN- positive cells in the CC	FST			OF first 5 minutes			OF total 20 minutes		
			Float	Swim	Strugg.	Total distance moved	Zone crossings	Duration in centre	Total distance moved	Zone crossings	Duration in centre
QUIN-positive cells in the CC	All mice	,569 *	,457	-,504	,443	,072	,121	-,052	,270	,212	-,223
	Males	,143	-,086	-,174	,486	,143	,143	-,029	,429	,314	,200
	Females	,679	,679	-,679	,464	,143	,214	,071	,429	,393	-,071
QUIN- immunoreactivity in the TRN	All mice	X	,242	-,294	,082	,440	,489	,011	,440	,478	-,407
	Males	X	,314	-,580	-,029	,771	,771	,657	,943 **	,886 *	,029
	Females	X	,393	-,393	,321	,429	,321	-,393	,536	,429	-,536

Figure 3. Left: positive correlation between QUIN-immunoreactivity in the TRN and QUIN-positive cells in the CC, $r_{sp}=0.569$, $p<0.05$, male $n=6$, female $n=7$. Right: positive correlation between track length in OF 20 minutes and QUIN-immunoreactivity in the TRN $r_{sp}=0.943$, $p<0.01$, male $n=6$.

4. Discussion

In the present paper, we show high levels of QUIN-immunoreactivity specifically in the CC and TRN of naïve mice. In the CC, QUIN-immunoreactivity delineated individual cells, whereas in the TRN the pattern of staining was more diffuse, resembling a fibrillary staining pattern. Interestingly, in males only, QUIN-immunoreactivity in the TRN showed a highly significant and very strong correlation with the measures of distance moved and zone crossings in the OF. Moreover, the number of QUIN-positive cells in the CC was positively correlated with QUIN-immunoreactivity in the TRN.

4.1 QUIN-positive cells in the CC

Specifically, QUIN-positive cells were observed in the CC and the RSC. Individual cells were observed with immunoreactivity specifically marking the cell membrane. The CC forms part of the limbic system, which is

associated with emotion processing, learning and memory (Hadland, Rushworth et al. 2003, Dalley, Cardinal et al. 2004, Kozlovskiy, Vartanov et al. 2012). The RSC is located behind the splenium of the corpus callosum and it is involved in episodic memory, navigation, imagination of future events and processing scenes (Vann, Aggleton et al. 2009). In humans, the RSC forms part of the posterior CC, together with Brodmann areas 23 and 31 (Maddock 1999, Vann, Aggleton et al. 2009). However, in rodents, the posterior CC is equivalent to only the RSC (Vogt, Vogt et al. 2004, Vann, Aggleton et al. 2009). It is reported that the RSC receives input from several areas of the CC (Maddock 1999, Vogt, Vogt et al. 2004). In addition, together with the posterior CC, the RSC has also been involved in emotion processing and memory (Maddock 1999, Vogt, Vogt et al. 2004). Thus, suggesting functions in common for both CC and RSC. The exact role of QUIN in these brain regions remains unclear. Interestingly, several studies reported impaired memory (Beninger, Jhamandas et al. 1986, Misztal, Skangiel-Kramska et al. 1996) and altered spatial reference memory (Shear, Dong et al. 1998) in rodents after injection of QUIN in the brain.

4.2 QUIN-immunoreactivity in the TRN

QUIN-immunoreactivity was observed in the TRN and partially disseminated to the zona incerta (ZI) in the hypothalamus. Here, we observed a slightly different, more diffuse staining pattern. The TRN is part of the ventral thalamus that contains gamma amino butyric acid (GABA)ergic cells. It is involved in the regulation of the sensory,

motor and limbic system as well as in thalamocortical communication (Pinault 2004, Lam and Sherman 2011). The ZI is a nucleus located ventral to the thalamus and contiguous with the TRN. Its exact function is still unknown, but it is suggested to be involved in the transmission of brainstem viscerosensory and somatosensory information (Power and Mitrofanis 2001). Interestingly, QUIN-immunoreactivity in males was strongly correlated to locomotion during 20 minutes' OF exposure, whereas we did not observe any correlation between QUIN-immunoreactivity and behavioural measures during the first 5 minutes of exposure to the OF. Thus, these data suggest a modulatory role for QUIN at the level of the TRN in mediating locomotion and possibly even hyperactivity, without affecting anxiety-related behaviour. Interestingly, the TRN has been implicated in attention-deficit hyperactivity disorder (ADHD), specifically in the modulation of arousal (di Michele, Prichep et al. 2005, Rowe, Robinson et al. 2005). Although highly speculative, this fact could explain the association that we found in males between increased QUIN-immunoreactivity and greater distance covered in the OF.

4.3 Correlation between the number of QUIN-positive cells in the CC and QUIN-immunoreactivity in the TRN

Interestingly, we found that the number of QUIN-positive cells in the CC was correlated to the degree of QUIN-immunoreactivity in the TRN. Evidence shows that the CC and the TRN are anatomically connected. The TRN receives projections from both the thalamus and cortex (Pinault

2004, Çavdar, Onat et al. 2008), which supports our finding of a correlation in QUIN-immunoreactivity between the CC and the TRN. In this study, the behavioural screening did not specifically address behavioral phenotypes directly related to the CC and TRN. In addition, the fact that no correlations were observed between QUIN-immunoreactivity in these two brain areas and behavioural performance in the FST in naïve mice, does not exclude a functional role for QUIN in the CC and TRN under challenging conditions, such as chronic inflammation or stress. Evidently, it would be highly interesting to more closely assess the exact role of QUIN function within the brain in view of e.g. emotion, learning, memory, navigation and hyperactivity. Moreover, as we already observe a role for QUIN in the CC and in the TRN in naïve mice, further research should also address the role of QUIN in these brain regions in models of stress and/or inflammation linked to depression.

4.4 Concluding remarks

As mentioned above, several neurotoxic effects have been attributed to altered QUIN levels, after injury or in patients with psychiatric disorders. In these cases, QUIN-immunoreactivity has mainly been reported in the hippocampus and cortex (Guillemin, Brew et al. 2005, Chung, Leung et al. 2009, Rahman, Ting et al. 2009, Chen, Stankovic et al. 2010). However, physiological levels of QUIN are required in normal, healthy conditions to produce nicotinamide adenine dinucleotide(NAD), which is needed for any cell to function properly (Myint 2012). In control samples of a number of studies, controversial results have been reported after evaluation of

QUIN-immunoreactivity. No QUIN-immunoreactivity was found in the rat cortex (Chung, Leung et al. 2009) and brain parenchyma of control rats (Moffett, Espey et al. 1993). In addition, another study showed no QUIN-immunoreactivity in the brains of sham-operated gerbils (Baratte, Molinari et al. 1998). On the other hand, when using human post-mortem control brain tissue, weak QUIN-immunoreactivity was detected in temporal neocortical and hippocampal areas (Guillemin, Brew et al. 2005, Guillemin, Cullen et al. 2007). In addition, QUIN-immunoreactivity has been shown in the cortex of control human brain tissue (Steiner, Walter et al. 2011). Amongst other things, species and strain differences as well as tissue processing and the use of different antibodies for staining for QUIN may explain the diversity of the results obtained.

We conclude that QUIN expression, particularly observed in the CC and TRN of naïve mice, and strongly correlated with locomotor activity in males, may play an important role at the level of the TRN in regulating motor behaviour. Evidently, it would be of great value to further explore the exact role of QUIN-immunoreactivity in mice in order to understand its neurotoxic effects in cases of injury, inflammation or in patients with psychiatric disorders.

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Chapter 5

Effects of prenatal Poly I:C exposure on global histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity in the mouse brain

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Submitted

Abstract

The aim of our study was to investigate the brain-specific epigenetic effects on global enzymatic histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity after prenatal exposure to maternal immune challenge by polyinosinic:polycytidylic acid (Poly I:C) at gestational day (GD) 17 in C57BL/6JRcchsd mouse offspring. Pregnant mice were randomly divided into 2 groups, receiving either 5 mg/kg Poly I:C or phosphate buffered saline (PBS) intravenously at GD 17. Subsequently, the effects on whole brain enzymatic HDAC and DNMT activity and the protein levels of various HDAC isoforms were assessed in the offspring. Overall, a significant sex x treatment interaction effect was observed after prenatal exposure to maternal immune challenge by Poly I:C, indicative of increased global HDAC activity particularly in female offspring from mothers injected with Poly I:C when compared to controls. Results on the levels of specific HDAC isoforms suggested that neither differences in the levels of HDAC1, HDAC2, HDAC3, HDAC4 or HDAC6 could explain the increased global HDAC activity observed in female Poly I:C offspring. In conclusion, we show that Poly I:C administration to pregnant mice alters global brain HDAC, but not DNMT activity in adult offspring, whereas it is still unclear which specific HDAC(s) mediate(s) this effect. These results indicate the necessity for further research on the epigenetic effects of Poly I:C.

1. Introduction

Infection during pregnancy and its associated maternal immune response may negatively impact upon offspring brain development (Gilmore and Jarskog 1997, Nawa, Takahashi et al. 2000), thereby increasing the risk to develop psychopathology later in life (Fatemi, Reutiman et al. 2008, Zhou 2012). The exact mechanisms by which prenatal maternal inflammation impacts upon offspring brain development remain to be elucidated though.

Several studies examined the effects of maternal infection during gestation in animal models using different triggers such as lipopolysaccharide (LPS) (Borrell, Vela et al. 2002, Golan, Lev et al. 2005), the human influenza virus (Fatemi, Reutiman et al. 2008) and polyinosinic:polycytidylic acid (Poly I:C). Poly I:C is a synthetic double stranded RNA frequently used as a viral mimetic that induces the release of pro-inflammatory cytokines (Doukas, Cutler et al. 1994, Snell, Chernyshev et al. 1997). As such, prenatal Poly I:C injection to the dams has been shown to induce various cognitive and behavioural changes in adult offspring (Ozawa, Hashimoto et al. 2006) including impairments in exploratory behaviour, prepulse inhibition, latent inhibition and spatial working memory (Meyer, Feldon et al. 2005). Moreover, moderate to severe cell loss was observed in the adult hippocampal CA1, CA3 and dentate gyrus after prenatal maternal exposure to Poly I:C (Zuckerman, Rehavi et al. 2003). As Poly I:C is known to induce a short-lasting immune response, the timing of the maternal inflammatory response can be accurately linked to specific windows during foetal development (Zuckerman, Rehavi et al. 2003). Interestingly, when Poly I:C was injected at

GD 6 or GD 9, impaired latent inhibition was observed in the offspring, whereas this effect was less pronounced when Poly I:C was injected at GD 13 and was even absent when injection occurred at GD 17 (Meyer, Feldon et al. 2006). In the literature, most of the studies use Poly I:C injection to the pregnant dams at GD 9, which roughly corresponds to the end of the first trimester of human pregnancy. Prenatal maternal infection during this period has mainly been associated with the development of schizophrenia (Brown, Begg et al. 2004, Li, Cheung et al. 2009, Sørensen, Mortensen et al. 2009). However, prenatal maternal immune challenge with Poly I:C at GD 17 has been linked primarily to the development of depressive-like behaviour in the offspring (Enayati, Solati et al. 2012, Babri, Doosti et al. 2014).

Despite the fact that this field of research has received increased attention, the exact mechanisms behind the association between maternal infection during pregnancy and altered brain development in the offspring have still not been elucidated. One likely candidate mechanism involved is epigenetic programming, which is known to play a critical role during fetal development. Furthermore, epigenetic processes modulate several functions in the adult nervous system, such as adult neurogenesis, synaptic plasticity, as well as cognition and emotionality (Ravi and Kannan 2013). The most well-known epigenetic processes include DNA methylation, histone modifications and noncoding RNAs. Epigenetic changes result in chromatin reorganization and thus regulate gene expression during e.g. cellular differentiation. More specifically, histone deacetylation, which generally leads to the compaction of chromatin and transcriptional repression, has

recently been linked to the pathophysiology of various psychiatric disorders. For example, changes in histone acetylation have been reported in animal models of depression and stress exposure (Yamawaki, Fuchikami et al. 2012). In addition to histone modifications, DNA methylation has also been shown to be involved in the development of depressive-like behaviours (Sales, Biojone et al. 2011), whereas both histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors have been reported to exert antidepressant effects (Sales, Biojone et al. 2011, Yamawaki, Fuchikami et al. 2012). In view of prenatal infection, hypoacetylation of histones H3 and H4 has been observed in the cortex of juvenile offspring prenatally exposed to Poly I:C (Tang, Jia et al. 2013). Moreover, HDAC inhibitors are known to impact upon the transcriptional regulation of cytokines, immunologic signaling pathways and the inflammatory response (Qureshi and Mehler 2011). To date, the epigenetic effects of prenatal maternal Poly I:C exposure at GD 17 –which, as stated above, links more closely to the development of a depressive-like phenotype– have not yet been studied.

In the present study, we examined the brain-specific global epigenetic effects of prenatal maternal immune challenge using viral mimetic Poly I:C at GD 17 in C57BL/6JRccHsd mouse offspring. We hypothesize that prenatal maternal Poly I:C exposure would lead to increased global HDAC and DNMT activity, which represents a molecular switch modulating the alterations in offspring brain development in response to prenatal maternal inflammation.

2. Material and methods

2.1 Animals

Experiments were conducted in accordance with permissions from the government and the veterinarian administration of Oberbayern (AZ: 55.2-1-54-2531-61-10). This study used C57BL/6JRccHsd mice obtained from Harlan Laboratories (Eystrup, Germany). Mice were housed 4 per cage in single-sex groups in individually ventilated cages (IVC; cage sizes 480 x 375 x 210 mm [depth x width x high]) under specific pathogen free (SPF) conditions and maintained on a 12/12h light-dark cycle (light on at 12AM) and temperature-controlled environment (relativity humidity $55\% \pm 5\%$; temperature: $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$; room air exchange rate: 15). Breeding food (Ssniff, Germany) and water were allowed ad libitum. Cages were changed every week. After transporting the mice to the local animal facilities, the habituation period lasted 3 weeks. During this period, mice were subcutaneously injected with a transponder for identification purposes. For mating, a male was added to a cage with a female for a period of 3 days. After mating, females were housed individually. Three weeks after birth, offspring were weaned and separated by sex (4 mice/cage). Offspring were chipped when they were 6 weeks old to facilitate their identification. A maximum of 2 mice/sex/litter were used to prevent litter effects (Chapman and Stern 1978).

2.2 Mating and induction of prenatal infection

Approximately 8 weeks after birth, male and female offspring were put together for 3 days. At GD17, pregnancy was confirmed by visual

inspection of the dam in combination with weight gain analysis (increase between day of mating and the day of the injection). Pregnant mice were randomly divided into 2 groups [Poly I:C; n=5] and phosphate buffered saline [PBS; n=6]). Dams were injected intravenously with 5 mg/kg Poly I:C (potassium salt, Sigma Aldrich (Germany)) dissolved in sterile water or an equivalent volume of PBS. Following injection, mice were observed daily until delivery.

2.3 Enzymatic HDAC and DNMT activity

At an age of thirteen weeks, brains from 8 male and 9 female mice prenatally exposed to Poly I:C, and 9 male and 9 female offspring from PBS-treated dams were used for epigenetic analysis. For this purpose, mice were anesthetized with a single intraperitoneal injection of Ketamin (Pfizer, Germany) and Xylacin (Bayer, Germany). 0.7 ml 10% Ketamin and 0.3 ml 2% Xylacin were mixed and administered at a dose of 10ml/kg. Afterwards, brains were collected and stored at -20°C in RNA-Later (Invitrogen, Germany). Half of the brain was used to extract nuclear proteins using a Nuclear Extract Kit (Active Motif, Belgium) and the activities of various HDAC and DNMT enzymes were analyzed with the HDAC Fluorescent Assay Kit and DNMT Activity/Inhibition Assay (Active Motif, Belgium). The fluorescence/optical density was detected with a PolarStar OPTIMA microplate fluorimeter (BMG Labtech).

2.4 Western blot

In order to elucidate which specific HDAC enzyme(s) would mediate the observed increase in overall HDAC activity (see below), a western blotting experiment was performed. For this purpose, the other half of each brain was crushed while frozen and stored at -80°C. Tissue was subsequently mechanically homogenized in the Mini-Beadbeater™ (Biospec, The Netherlands) using lysis buffer (PBS, 1% Igepal CA-630®, 0.1% triton, 1% glycerol, 1mM EDTA, 1mM EGTA) and protease inhibitors (Complete Protease Inhibitor Cocktail, Roche Diagnostics, The Netherlands) in Milli-Q. Concentration of total protein was measured using the Lowry protein assay (Bio-Rad, The Netherlands). Proteins dissolved in sample buffer (1M Tris HCl pH 6.8, 75% glycerol, SDS, β -mercaptoethanol, bromophenol blue in Milli-Q) were denatured by boiling at 100°C for 7 minutes. Thirty microgram of protein per lane, in case of HDAC4 and 6, and 100 μ g for HDAC1, 2 and 3 was separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 75 min at 150V and transferred to a nitrocellulose membrane during 90 min at 100V using a Bio-Rad Laboratories Western Blotting system. Next, membranes were rinsed once in PBS and blocked in 1:1 Odyssey Blocking Buffer:PBS (LICOR Biosciences, NE, USA) for 1 hour at room temperature. Following the blocking of the membranes, they were incubated overnight at 4°C with the respective monoclonal mouse anti-mouse HDAC1 (1:350), HDAC2 (1:500), HDAC3 (1:350) and monoclonal anti-rabbit HDAC4 (1:500) contained in the HDAC Antibody Sampler Kit (#9928, Cell Signaling Technology, Inc.) or the monoclonal anti-rabbit HDAC6 (1:500) (#7612,

Cell Signaling Technology, Inc.) and 1:1000 diluted monoclonal mouse anti- β -actin (sc-81178, Santa Cruz Biotechnology) diluted in either 1:1 Odyssey Blocking Buffer:PBS (HDAC1, 3, 4) or 1:1 Blocking buffer:PBS + 0,2% Tween-20 (HDAC2 and 6). After incubation with the primary antibody, membranes were rinsed once with PBS:Tween and twice with PBS and then incubated with the corresponding secondary antibody, i.e., Alexa Fluor® 680 donkey anti-mouse (1:10000) IgG or Alexa Fluor® 800 goat anti-rabbit IgG (1:5000; LI-COR Biosciences) diluted in 1:1 Blocking buffer:PBS for 1 hour at room temperature. Membranes were rinsed again as explained before and bands were visualized by enhanced chemiluminescence detection using an Odyssey Scanner and the Odyssey Infrared Imaging System v2.1 software (LI-COR Biosciences). Bands were quantified with the ImageJ software (NIH, USA) using mean intensity and normalizing for β -actin expression.

2.5 Statistical analysis

The effects of prenatal maternal Poly I:C exposure on enzymatic HDAC and DNMT activity and protein levels of each HDAC isoform were first subjected to a two-way analysis of variance (ANOVA; treatment x sex) with IBM SPSS Statistics version 20 (SPSS Inc., USA). Significant interaction effects were analyzed in more detail using post-hoc LSD tests. The level of significance was set at $p < 0.05$ in all cases. Graphs were designed with GraphPad Prism 6.

3. Results

3.1 Global enzymatic activity

Regarding HDAC activity, a significant sex x treatment interaction effect was found ($F_{3,31}=5.477$; $p<0.05$) (see Figure 1). Post hoc LSD tests indicated that HDAC activity tended to be increased in female offspring from mothers injected with Poly I:C ($p=0.064$) compared to those injected with PBS. No overall effects were found for sex or treatment ($F_{3,31}=0.884$ and $F_{3,31}=0.110$ respectively; both $p>0.05$). For total DNMT activity, no significant effects were detected ($F_{3,31}=1.676$, $F_{3,31}=0.826$, $F_{3,31}=0.159$ for sex, treatment and their interaction, respectively; all $p>0.05$) (see Figure 1).

Figure 1. HDAC and DNMT activity levels in mouse offspring. Bars represent means + S.E.M, # $p<0.05$ (ANOVA, sex x treatment), (*) $p=0.064$ (LSD). Vehicle male $n=9$, vehicle female $n=9$, Poly I:C male $n=8$, Poly I:C female $n=9$. For total DNMT activity no effects were detected ($F_{3,31}=1.676$, $F_{3,31}=0.826$, $F_{3,31}=0.159$; all $p>0.05$).

3.2 HDAC isoforms

Overall, a sex effect was observed with reduced HDAC1 levels in female versus male mice ($F_{3,31}=4.389$; $p<0.05$). Furthermore, a tendency for an overall treatment effect was shown for HDAC6 with increased protein

levels in Poly I:C offspring when compared to control offspring ($F_{3,29}=4.061$; $p=0.053$). No effect was found for HDAC2, HDAC3, and HDAC4 (all $F_{3,31}<3$; $p>0.05$) (see Table 1 and Figure 2).

Table 1. Mean and S.E.M for each condition for the correspondent HDAC

	male vehicle		male Poly I:C		female vehicle		female Poly I:C		effects
	M	SEM	M	SEM	M	SEM	M	SEM	
HDAC1	0.1655	0.0402	0.2027	0.0510	0.1195	0.0350	0.0875	0,0264	sex effect $p<0.05$
HDAC2	0.2518	0.0353	0.1874	0.0285	0.2362	0.0278	0.2620	0.0475	
HDAC3	0.0735	0.0262	0.0502	0.0323	0.0506	0.0125	0.0501	0.0313	
HDAC4	0.1303	0.0322	0.1194	0.0335	0.1005	0.0241	0.1178	0.0296	
HDAC6	0.1277	0.0386	0.3348	0.1242	0.1731	0.0563	0.2479	0.0684	treatment effect $p<0.1$

Figure 2. Levels of HDAC1, HDAC2, HDAC3, HDAC4 and HDAC6 proteins in brain homogenates and corresponding quantification. Bars represent means + S.E.M, \$ $p < 0.05$ (ANOVA, sex), † $p < 0.1$ (ANOVA, treatment). HDAC1-5: Vehicle male $n=9$, vehicle female $n=9$, Poly I:C male $n=8$, Poly I:C female $n=9$. HDAC 6: Vehicle male $n=9$, vehicle female $n=9$, Poly I:C male $n=6$, Poly I:C female $n=9$.

4. Discussion

In the present paper, we report that prenatal maternal Poly I:C exposure in the mouse modulates global brain HDAC activity, but not DNMT activity in adult offspring. HDAC activity was particularly increased in female offspring exposed to prenatal maternal Poly I:C when compared to controls, suggesting that epigenetic programming may, at least in part, mediate the long-term effects of prenatal maternal infection.

Exposure to pathogens triggers an inflammatory response with concomitant up-regulation of pro-inflammatory cytokines. Interestingly, next to the fact that HDACs were shown to regulate gene transcription of cytokines (Lu, Sun et al. 2005, Villagra, Sotomayor et al. 2009), cytokines have recently also been shown to regulate the activity of HDACs. For example, activation of pro-inflammatory cytokines, such as tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), produces HDAC1 ubiquitination and proteosomal degradation (Villagra, Sotomayor et al. 2009). Apart from the modulation of innate immune system function, HDACs also regulate adaptive immunity (Shakespeare, Halili et al. 2011). Depletion of both HDAC1 and HDAC2, specifically in B cells leads to blocking of early B cell development (Yamaguchi, Cubizolles et al. 2010). Over the last years, increased evidence linked histone acetylation to the neurobiology and pharmacology of mood disorders (Zarate, Singh et al. 2006). For instance, chronic defeat stress resulted in an impaired behavioural response and in the downregulation of brain-derived neurotrophic factor (BDNF) transcripts III and IV, which was reversed by imipramine. Interestingly, this antidepressant also increased histone acetylation at these promoters and was associated with a downregulation of HDAC5 (Tsankova, Berton et al. 2006). Vice versa, HDAC inhibitors have also been shown to possess antidepressant properties (Covington, Maze et al. 2009, Grayson, Kundakovic et al. 2010). For example, administration of the HDAC inhibitor sodium butyrate alone or in combination with fluoxetine decreased immobility scores in the tail suspension test, a measure for behavioural despair, in mice (Schroeder, Lin et al. 2007). However, these studies used naïve mice that were not exposed

to a prenatal immune challenge. As mentioned above, the effects produced by prenatal maternal infection are depending on the timing of exposure during gestation and their behavioural expression may also depend on the age of the offspring. For instance, after Poly I:C injection at GD 9, global histone H3 and H4 hypoacetylation was shown in in the cortex of juvenile but not adult offspring (Tang, Jia et al. 2013). Our results are partially consistent with this study, e.g. we found that HDAC activity was increased in offspring that were exposed to prenatal maternal Poly I:C (at GD 17), an effect that seemed to be particularly pronounced in females. However, our findings were observed in adult offspring, whereas Tang and colleagues detected hypoacetylation only in juvenile offspring, a difference that may be explained by the different timing of prenatal maternal Poly I:C exposure.

We hypothesized that the observed increase in global HDAC activity in females, which were prenatally exposed to maternal Poly I:C, would be reflected in enhanced expression of one of the HDAC proteins assessed. However, the western blot showed that none of the HDAC enzymes examined was present in significantly higher levels in Poly I:C versus control female offspring. Although we observed a treatment effect with increased HDAC6 in offspring of mice treated with Poly I:C compared to controls, this effect seemed to be more pronounced in male – and not female – offspring, which does not match the global HDAC activity data.

In the brain, HDAC6 is involved in protein aggregate elimination, in neuronal oxidative stress and in the mitochondrial transport (Simoes-Pires, Zwick et al. 2013). Interestingly, HDAC6 deficiency in mice resulted in hyperactivity

and reduced anxiety, and depression-like behavior. Similarly, administration of an HDAC6 inhibitor also had an antidepressant-like effect, suggesting a role for HDAC6 in the expression of emotional behaviours (Fukada, Hanai et al. 2012). As commented above, prenatal maternal Poly I:C exposure has been associated with the development of depressive-like behaviour in the offspring. Interestingly, we observed that HDAC6 protein levels tended to be increased in Poly I:C offspring when compared to control offspring. Altogether, though purely speculative, these data suggests a role for HDAC6 in mediating the association between prenatal maternal infection and the development of depression in the offspring. Thus, further research investigating this possible role of HDAC6, would be of great value.

In addition, we observed a sex effect with reduced HDAC1 levels in female mice compared to males. Regarding brain functions, HDAC1 is reported as the molecular switch between neuronal survival and death (Bardai, Price et al. 2012) and being considered as a negative regulator of fear extinction in mice (Bahari-Javan, Maddalena et al. 2012) and as a negative regulator of mood (Schroeder, Lewis et al. 2013). No studies have examined sex differences in HDAC1 function in this respect. Thus, future research should address possible variations in HDAC1 levels between sexes.

A feasible explanation for the discrepancy observed between the global HDAC activity and the protein levels of several HDAC isoforms in the present study is that one or more other HDAC isoform(s) than those assessed using western blotting is/are involved in mediating the observed increased in global HDAC activity. HDACs are part of a large family of proteins, with 11 members identified to date. We examined HDAC1, HDAC2, HDAC3, HDAC4

and HDAC6, because of the evidence showing their association with mental (dys)function (McQuown, Barrett et al. 2011, Bahari-Javan, Maddalena et al. 2012, Fukada, Hanai et al. 2012, Morris, Mahgoub et al. 2013, Sarkar, Chachra et al. 2014). A viable candidate that may contribute to the effect found in HDAC global activity is HDAC5, which has been shown to be involved in inflammation, specifically in activated monocytes and macrophages (Cantley and Haynes 2013). Moreover, decreased HDAC5 levels in the hippocampus has been associated to the antidepressant effects of imipramine in mice models, as explained above (Tsankova, Berton et al. 2006). We initially included HDAC5 in the present study, but after trying numerous different antibodies in several organ tissues, we were not able to detect a specific band indicating HDAC5. Another, completely different possible explanation for the observed discrepancy may lie in the fact that an increased enzymatic activity does not necessarily imply an increase in the level of the associated protein(s). Clearly, this issue awaits further investigation.

4.1 Conclusions

Altogether, this study indicates that prenatal maternal Poly I:C exposure seems to increase global HDAC, but not DNMT activity, particularly in female offspring. Evidently, it would be of great value to further explore the exact role of epigenetic programming in response to prenatal exposure to maternal immune challenge using Poly I:C in order to understand the mechanism underlying the association between prenatal infection/inflammation and adult psychopathology.

4.2 Limitations

It should be noted that mice were anesthetized in this study in order to comply with the approval of the ethics committee. Although the time window between exposure and actual euthanasia was extremely short in our case, we cannot fully exclude the possibility that the use of anesthesia may have influenced epigenetic processes (Lirk, Fiegl et al. 2014). In addition, it should be noted that for assessing both HDAC levels and activity, whole brain homogenates were used. It would evidently be of additional interest to evaluate specific brain areas and cell-type-specific epigenetic changes.

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Chapter 6

Depressive symptoms during IFN-alpha therapy in humans associated with changes in the kynurenine pathway

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Abstract

Although Interferon-alpha (IFN- α) is effective for the treatment of Hepatitis C, this therapy has been linked to the development of depressive symptoms and major depression in a substantial proportion of patients. This may be mediated by neurotoxic components of the kynurenine pathway. The aim of our study was to investigate the effects of IFN- α therapy for Hepatitis C on the Montgomery-Asberg Depression Rating Scale (MADRS), the Beck Depression Inventory (BDI) and on several enzymes and metabolites of the kynurenine pathway.

Twelve patients diagnosed with Hepatitis C were followed for 48 weeks during treatment with IFN- α and ribavirin. During this period, symptom development was measured and blood samples were obtained at 12 regular time points. Relative gene expression analysis of the above mentioned enzymes was performed by real time quantitative polymerase chain reaction (RT-qPCR) using mRNA derived from blood cells. Metabolites were analysed in serum using a LC-MS/MS system.

During IFN- α therapy, we observed a significant change over time in MADRS and BDI scores. In addition, levels of the neurotoxic metabolite kynurenine (KYN), 3-hydroxykynurenine (3HK), quinolinic acid (QUIN) and the kynurenine/tryptophan (KYN/TRP) ratio were increased over time, while the kynurenic acid/kynurenine (KYNA/KYN) ratio, reflecting neuroprotective properties, was reduced. Regarding the enzymes of the kynurenine pathway kynureninase (KYNase), kynurenine-3-monooxygenase (KMO) and kynurenine aminotransferase (KAT-3) activity were also increased. When assessing the relationship between MADRS scores and metabolites, KYNA

and the KYNA/KYN ratio were negatively associated with MADRS scores. There was no association between enzymes and the MADRS score. Finally, relative expression of KYNase significantly predicted levels of 3HK.

We show that the kynurenine pathway plays an important role in the development of depressive symptoms during IFN- α treatment, with a shift in the balance between neurotoxic and neuroprotective metabolites, as well as increased expression of enzymes regulating the neurotoxic arm of the pathway.

1. Introduction

Interferon-alpha (IFN- α) is a commonly used cytokine to treat hepatitis C. However, several side effects have been reported, such as fever, fatigue and myalgias in a high percentage of patients, reviewed by (Foster 2010). In addition, full-blown neuropsychiatric syndromes, in particular clinical depression, have been observed (Schaefer, Hinzpeter et al. 2007), and often necessitates interruption or even cessation of the treatment (Debien, De Chouly De Lenclave et al. 2001). Various mechanisms have been suggested to underlie the pro-depressive effects of IFN- α (for review see Hoyo-Becerra, Schlaak et al. 2014). Of particular interest is the induction of pro-inflammatory cytokines, of which the circulating levels are associated with depressive symptoms during IFN- α treatment (Wichers, Kenis et al. 2007). Pro-inflammatory cytokines and other inflammatory processes can impact on brain function, disturb affect regulation and can lead to depression via diverse mechanisms (Capuron and Miller 2011, Haroon, Raison et al. 2012). Recently, an array of studies points towards an enhanced catabolism of tryptophan along the kynurenine pathway as being a major mediator in the association between inflammation and depression. Cytokines are known to stimulate the enzyme indoleamine 2,3-dioxygenase (IDO) that catabolizes tryptophan to kynurenine (Myint 2012). Thus, an increase in cytokines will result in a reduction of tryptophan (TRP) and will increase kynurenine (KYN). Concomitantly serotonin synthesis will be compromised because of reduced TRP availability (Asnis and De La Garza 2006). KYN can be further metabolized either to kynurenic acid (KYNA) by kynurenine aminotransferases (KAT) -1, KAT-2 and KAT-3, or to quinolinic acid (QUIN)

136

through several enzymatic reactions. First, KYN is metabolized to 3-hydroxykynurenine (3HK) by kynurenine-3-monooxygenase (KMO). Next, 3HK is further degraded to 3-hydroxyanthranilic acid (3HAA) by kynureninase (KYNase). Finally, 3HAA is degraded into QUIN by 3-hydroxyanthranilic acid oxygenase (3HAO). QUIN is further catabolized into nicotinamide adenine dinucleotide (NAD) by quinolinate phosphoribosyl transferase (QPRT) (Bender and McCreanor 1985, Heyes, Achim et al. 1996).

Activation of IDO is hypothesized to play an important role in the development of major depression, either via TRP depletion and the consequent reduction in the synthesis of serotonin, or via activation of the KYN pathway and the synthesis of the KYN metabolites (Christmas, Potokar et al. 2011). Some of these, e.g. QUIN, are known to exert neurotoxic effects (Garthwaite and Garthwaite 1987, Khaspekov, Kida et al. 1989). Several studies have evaluated the levels of TRP, KYN and serotonin in depressed patients, but reported controversial results (Hughes, Carballedo et al. 2012, Myint, Bondy et al. 2013).

To our knowledge, in IFN- α induced depression only IDO activity has been analyzed from the several enzymes forming part of the KYN pathway. Its activity was assessed by means of the KYN/TRP ratio (Capuron, Neurauter et al. 2003, Comai, Cavalletto et al. 2011). Gene expression levels of enzymes in the KYN pathway have not yet been examined. The present study was undertaken to longitudinally evaluate: 1) the serum levels of TRP, KYN, 3HK, KYNA, QUIN, and 5-Hydroxyindoleacetic acid (5-HIAA) and their ratios, and 2) the gene expression levels of the KYN pathway enzymes IDO-1, KAT-1, and

KAT-3, KMO, KYNase, 3HAO and QPRT in blood of Hepatitis C patients treated with IFN- α . The metabolite ratios were calculated as indicators of IDO-activity and activation of the KYN pathway (KYN/TRP), activation of the neurotoxic arm (QUIN/KYN) and activation of the neuroprotective arm (KYNA/KYN) of the pathway. Finally, association of metabolites and enzyme expression levels with depressive symptoms were examined. To our knowledge, this is the most comprehensive study on the KYN pathway in IFN- α induced depression, and the first to examine the enzymes involved. We hypothesize that treatment with IFN- α induces depressive symptoms, which are associated with changes in the levels of the metabolites and the gene expression of the enzymes of the KYN pathway, which represent possible molecular candidates involved in depression.

2. Materials and methods

2.1 Subjects

Twelve patients (seven male (58%) and five female (42%) with a mean age of 45.75 years) diagnosed with chronic hepatitis C infection (HCV) were recruited from the Maastricht University Medical Center (MUMC) in the Netherlands, and assessed before and during standard treatment with pegylated IFN- α and ribavirin. Chronic HCV was defined as: positive for HCV-antibodies, HCV-RNA positive and elevated levels of transaminases at least once in the preceding six months.

The exclusion criteria for participation were age under 18 years and patients with a diagnosis of cancer, cerebrovascular disease, organic

psychiatric disease and active drug abuse that may influence the research outcome. In addition, patients meeting criteria after a psychiatric interview for mental disorders such as schizophrenia, bipolar disorders, mental retardation and dementia among other neurodegenerative disorders were excluded. Lastly, analphabetism and other disorders that under the point of view of the researcher may influence the investigation were also excluded.

The study was approved by the Committee of Maastricht University Medical Center. Each subject signed a written informed consent prior to participation.

2.2 Measurements

A total of 12 measurements were performed during the study that lasted 48 weeks. The first time point corresponded to the baseline measurements, which were assessed 2-4 weeks before starting treatment. Next, IFN- α treatment started and measurements were taken every 4 weeks to monitor and measure symptom severity. At each assessment, venous blood was collected and severity of depressive symptoms was determined using the Montgomery-Asberg Depression Rating Scale (MADRS), by an independent research assistant, and the Beck Depression Inventory (BDI) by means of self-reporting.

Blood samples were collected and serum prepared according standard procedures. Samples were stored at -80°C. For gene expression analyses, blood was collected in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). RNA was extracted using the Blood RNA Kit

(PreAnalytiX GmbH, Hombrechtikon, Switzerland) according to manufacturer's instructions and stored at -80°C.

2.3 Gene expression analysis

First strand cDNA synthesis kits (Thermo scientific, Lithuania) were used to prepare cDNA. Relative gene expression analysis was performed by real time quantitative polymerase chain reaction (qPCR) using the Lightcycler 480 Real-Time PCR System (Roche Applied Science, IN, USA) and the ABI 7900 (Applied Biosystems, Singapore).

After incubation for 5 min at 95°C, DNA amplification was performed over 40 cycles at 15 sec at 95°C, followed by a 30 sec at both 60°C and 72°C in the lightcycler. In the ABI the conditions for the reaction involved incubation for 10 min at 95°C, and 40 cycles in a fixed sequence of 15 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C. The melting curve was run at the end to confirm the identity and purity of the PCR. Primers sequences are presented in Table 1. The housekeeping genes RS27 alpha, PPIB1 and β -actin were compared in order to determine their stability. For this purpose, using a webtool (<http://www.leonxie.com/referencegene.php>), four different methods, e.g. Delta CT, Bestkeeper, Normfinder and Genorm were applied and determined RS27 alpha as the most stable gene. Thus, gene expression was normalized using RS27 alpha as reference gene. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also employed for interplate reference. Fold changes (FC) and relative gene expression values were calculated with the Pfaffl method.

Table 1. Primer design for qPCR

Genes	Forward primer	Reverse primer
IDO-1	GCAGTAAAGGATTCTTCTGGTC	GCATTTGCATTGCCTTGAA
KAT-1	TTCATGCTTAACCAGTACACCAA	CTGAGCGGGTCTATCTCCTG
KAT-3	GAGAAGTTTGTGCGTTTTTGC	CACTCCATGCCTTGATGATTT
KMO	CCAGAAATACTTCCGGATGC	AGTGAAATGAAGAGCACTTTACAGAT
KYNase	AGAACTAGATAAGTGGGCCAAAATAG	CTCTCATCTCCTGTAATCCAAGG
3HAO	TGTTTGGGGACACCTATGAGA	CATTGTCACCACCGAGGAG
QPRT	GGACAACCTCAAGCCAGAGG	GGTTGTCCAGGGTGATGC
ActB	GTTGTCGACGACGAGCG	GCACAGAGCCTCGCCTT
PPIB1	GTTTGAAGTTCTCATCGGGG	AAAACAGCAAATCCATCGTG
RS27 alpha	GGTTAAGCTGGCTGTCCTGAA	AGAAGGGCACTCTCGACGAA
GAPDH	CCCCGGTTTCTATAAATTGAGC	CACCTTCCCCATGGTGTCT

2.4 Kynurenine metabolites analysis

2.4.1 Instrumentation

The chromatographic system was composed of a Waters Acquity UPLC separations module connected to a Xevo TQ MS triple-quadrupole mass spectrometer, equipped with a Z-spray ESI ion source (Waters Corp., Milford, MA, U.S.). Separation was carried out using a Kinetex XB-C18, 2.6 μm , 2.1 x 150 mm column (Phenomenex, Torrance, California, U.S.).

2.4.2 Chemicals

Reagents for protein precipitation, derivatisation, and chromatography were purchased from Sigma-Aldrich (St. Louis, MO, U.S.), and Biosolve (Valkenswaard, NL).

2.4.3 Standards and control samples

TRP, KYN, 3HK, KYNA, QUIN, and 5-HIAA were purchased from Sigma-Aldrich (St. Louis, MO, U.S.). Standards and controls were established by adding defined volumes of the stock solutions to a human serum obtained from a blood bank. Quality control samples were generated to obtain low and high amounts of the analytes.

2.4.4 Sample extraction

A total sample volume of 300 μ l serum was used. Analytes were extracted from samples and calibrators/controls by adding 50 μ l of 2.0 M urea and 50 μ l of internal standard solution containing KYNA-D5, PIC-D4 and TRP-D5. Two precipitation steps by subsequently adding methanole/ethanol and CAN were carried out. The supernatant was separated into two portions, which were evaporated separately. One of these portions was directly reconstituted in mobile phase, while the other portion was used for derivatisation by addition of HCl/Butanol. After evaporation to dryness, this portion was reconstituted in mobile phase, too.

2.4.5 Chromatographic conditions

7.5 μ l of the reconstituted samples/calibrators/controls were loaded onto the LC-MS/MS system. 3HK and QUIN were analysed from the derivatized sample, while all other analytes were determined from the underivatized sample. A gradient method with a total duration of 7.5 min was used for chromatographic separation. Mobile phase A was composed of 0.1% formic acid and 0.01% HFBA in water; mobile phase B was methanol. Flow rate was set at 0.25 ml/min, column temperature was set at 30.0 °C. Retention times for the analytes were between 3.1 and 6.0 min.

2.4.6 MS condition

The Xevo TQ MS was operating in atmospheric pressure electrospray ionization (ESI) positive mode (ESI+). Ion source settings were: capillary voltage, 1.00 kV; desolvation temperatures, 650 °C; source temperature, 150 °C; nitrogen was used as desolvation gas with a cone gas flow rate of 1200 l/h; argon was used as collision gas at a flow rate of 0.15 ml/min. The analytes and internal standards were detected using multi reaction monitoring (MRM) technique. System operation, data acquisition and data processing were controlled using MassLynx V4.1 software (Waters, Milford, USA).

2.5 Statistical analysis

The statistical software program STATA (version 13.1, Statacorp, Texas, USA) was used for the analysis of the data. The metabolites TRP, KYN,

3HK, KYNA, QUIN, and 5-HIAA, and the gene expression levels of the enzymes IDO-1, KAT-1, and KAT-3, KMO, KYNase, 3HAO and QPRT were analyzed. In addition, the ratios KYN/TRP, QUIN/KYN and KYNA/KYN were calculated. To improve normality of distributions the QUIN/KYN ratio was subjected to an inverse square root transformation, the enzymes KMO and KYNase and the metabolites 3HK, 5HIAA and KYNA were subjected to square root transformation and QPRT, IDO1 and KAT1 were log transformed.

Multilevel mixed-effects linear regression analysis was applied using the XT MIXED command of Stata. Effect sizes of explanatory variables were expressed as regression coefficients. Where appropriate, a squared term of time was added to the model (β_1 : linear, β_2 : square), due to non-linear changes of certain variables overtime. The level of significance was set at $p < 0.05$ in all cases. Analyses were corrected for age, gender and use of antidepressants, benzodiazepines and opioids during the study. Graphs were designed with Microsoft Excel.

First, effects of IFN- α treatment over time on the depression scores MADRS and BDI, on metabolite levels or their ratios, as well as on relative gene expression values were analyzed. Since both depression rating scales, MADRS and BDI were highly correlated (coef. 0.788, $p < 0.0001$) and MADRS is used frequently to assess IFN- α induced depressive symptoms in other studies, MADRS score was regressed on the different metabolites and relative gene expression values of the enzymes to assess the association between the occurrence of depressive symptoms and metabolites or enzyme expression respectively. Last, associations

between metabolites and expression of enzymes of the KYN pathway were examined.

3. Results

3.1 Effect of IFN- α treatment on depression scores

A significant change over time was observed for the MADRS ($\beta_1 = 2.3689$, $p < 0.001$; $\beta_2 = -0.1824$, $p < 0.001$) and BDI depression scores ($\beta_1 = 1.6639$, $p = 0.001$; $\beta_2 = -0.1129$, $p = 0.003$), during IFN- α treatment. While depressive symptoms steadily increased during the first 6 months of treatment, they gradually decline again in the last 4 months (see Figure 1). Post-hoc analysis indicated that MADRS scores were significantly higher from baseline at time points 3, 4, 5, 6 and 8; BDI scores were increased at time points 3, 5, 6, 7, 8, 10 and 11.

Figure 1. Change over time in MADRS and BDI scores during IFN- α treatment. See text for details.

3.2 Effect of IFN- α treatment on kynurenine metabolites

A significant increase of KYN ($\beta_1 = 12.4363$, $p=0.024$), 3HK ($\beta_1 = 0.1049$, $p=0.001$) and QUIN ($\beta_1 = 2.8347$, $p=0.005$) over time were observed (see Table 2 and Figure 2).

Table 2. Effect of IFN- α treatment over time on some metabolites of the kynurenine pathway and ratios

Metabolites	β	p-value
TRP	-133.9359	0.375
KYN	12.4363	0.024
3HK	0.1049	0.001
QUIN	2.8347	0.005
KYNA	0.0265	0.139
5-HIAA	0.0173	0.673
Ratios	β	p-value
KYN/TRP	0.0011	0.000
QUIN/KYN	0.0042	0.694
KYNA/KYN	$\beta_1 = -0.0011$	0.000
	$\beta_2 = 0.0001$	0.001

Figure 2. Change over time in serum levels of KYN, 3HK and QUIN during IFN- α treatment. See text for details.

The KYN/TRP ratio ($\beta_1 = 0.0011$, $p < 0.001$) significantly increased over time. The KYNA/KYN ratio also significantly changed over time ($\beta_1 = -0.0011$, $p < 0.001$; $\beta_2 = 0.0001$, $p = 0.001$): it gradually decreased the first 6 months and increased again thereafter (see Table 2 and Figure 3). Post-hoc analyses showed that the KYNA/KYN ratio was significantly lower from baseline on time points 5, 6, 7, 8 and 9.

Figure 3. Change over time in the KYN/TRP and the KYNA/KYN ratios during IFN- α treatment. See text for details.

3.3 Effect of IFN- α treatment on enzymes of the KYN pathway

KYNase ($\beta_1 = 0.0057$, $p < 0.036$) was significantly increased over time during IFN- α treatment. In addition, a significant change over time was observed on KMO ($\beta_1 = 0.0312$, $p < 0.001$; $\beta_2 = -0.0021$, $p < 0.001$) and KAT-3 ($\beta_1 = 0.0140$, $p < 0.001$; $\beta_2 = -0.0010$, $p < 0.001$). Post hoc analysis indicated that IFN- α therapy increased KYNase and KMO expression in all time points, and KAT-3 in all but not 2 and 12 (see Table 3 and Figure 4).

Table 3. Effect of IFN- α treatment over time on some enzymes of the kynurenine pathway

Enzymes	β	p-value
IDO1	0.0258	0.159
KYNase	0.0057	0.036
KMO	$\beta_1 = 0.0312$	0.000
	$\beta_2 = -0.0021$	0.000
3HAO	0.0000	0.901
QPRT	0.0115	0.615
KAT1	0.0199	0.120
KAT3	$\beta_1 = 0.0140$	0.000
	$\beta_2 = -0.0010$	0.000

Figure 4. Change over time in KYNase, KMO and KAT-3 during IFN- α treatment. See text for details.

3.4 Association between MADRS depression scores and metabolites

KYNA was negatively associated with the MADRS score ($\beta_1 = -3.4894$, $p = 0.029$). In addition, a significant association was found between the ratio KYNA/KYN over time with the MADRS score ($\beta_1 = -646.1161$, $p = 0.008$). Associations between the rest of metabolites, e.g. KYN, QUIN, TRP, 3HK, 5-HIAA, and MADRS scores did not reach significance (see table 4).

Table 4. Association between metabolites, ratios and symptom severity measured using MADRS

Metabolites	β	p-value
TRP	-0.0003	0.141
KYN	-0.0039	0.499
3HK	-0.2068	0.863
QUIN	-0.0346	0.268
KYNA	-3.4894	0.029
5-HIAA	-0.0088	0.991
Ratios	β	p-value
KYN/TRP	-58.4424	0.609
QUIN/KYN	-0.1979	0.943
KYNA/KYN	-646.1161	0.008

3.5 Association between MADRS depression scores and enzymes

MADRS scores were not associated with any of the examined KYN pathway related genes (see table 5).

Table 5. Association between enzymes and symptom severity measured using MADRS

Enzymes	β	p-value
IDO1	-0.9818	0.303
KYNase	4.9131	0.419
KMO	17.6553	0.147
3HAO	-202.0669	0.737
QPRT	-0.2137	0.765
KAT1	1.2232	0.345
KAT3	16.3008	0.360

3.6 Association between metabolites and enzymes

We examined whether levels of KYN pathway metabolites could be predicted by the expression levels of the respective enzymes. Specifically, associations between the relative gene expression of the enzyme and its substrate and its product were analysed. 3HK was found positively associated with KYNase ($\beta_1 = 1.9877$, $p = 0.030$). No other significant associations were observed between metabolites and enzymes of the KYN pathway (see Table 6).

Table 6. Association between metabolites and enzymes of the kynurenine pathway (β_1 and p-value)

	TRP	KYN	KYNA	3HK	QUIN
IDO1	-119.5412	62.6987			
	0.878	0.219			
KAT1		-43.1568	-0.1295		
		0.200	0.162		
KAT3		527.8825	0.8131		
		0.332	0.623		
KMO		488.5556		2.9310	
		0.134		0.068	
KYNase		93.7838		1.9877	
		0.625		0.030	
QPRT					3.1458
					0.438

4. Discussion

To the best of our knowledge, this is the most detailed study regarding longitudinal alterations in the KYN pathway in association with depressive symptoms during the course of IFN- α treatment. We report that, during a 12 month period of IFN- α treatment, depressive symptoms, as measured by the MADRS and BDI questionnaires, first steadily increase and after 6 months gradually decrease again. In addition, IFN- α therapy increased neurotoxic metabolites of the KYN pathway, e.g KYN, 3HK and QUIN, which was also reflected in changes of the KYN/TRP and KYNA/KYN ratios over time. Interestingly, a negative association was observed between the neuroprotective indicators (KYNA and the KYNA/KYN ratio) and the MADRS score. We also examined for the first time longitudinal changes in expression levels of genes involved in the KYN pathway. Relative expression of KMO, KYNase and KAT-3 was increased over time during IFN- α treatment. Finally, the enzymes KYNase and 3HK were significantly associated.

As mentioned above, one of the most effective treatments for hepatitis C is IFN- α . However, a considerable number of patients undergoing this therapy develop clinical depression (Asnis and De La Garza 2006, Papafragkakis, Rao et al. 2012). In line with our results, several studies reported higher scores for depression after IFN- α therapy (Raison, Dantzer et al. 2010, Shakoor, Shafqat et al. 2010, Pavlovic, Delic et al. 2011, Medeiros, Kayo et al. 2014). In these reports, the duration of IFN- α was differed from 12 until 48 weeks. Commonly, depression scores are measured only at the end of the treatment or at the most, on 4 time points: at baseline, 12th, 24th and 48th

week. When comparing different time points, higher depression scores are observed mainly in the 12th and 24th week. In our study, depression scores were measured every 4 weeks during 48 weeks. Moreover, in line with the studies mentioned, we observe the highest levels of depressive symptoms after 24 (MADRS score) and 28 weeks (BDI score). We also observe a decrease in the MADRS and BDI score from week 32 on. Medeiros *et al.* reported a reduction in the BDI scores after 48 weeks of IFN- α treatment (Medeiros, Kayo *et al.* 2014). Interestingly, administration of several antidepressants have shown to relieve IFN- α induced depression (Sockalingam and Abbey 2009, Baraldi, Hepgul *et al.* 2012). Mainly the effects of the selective serotonin reuptake inhibitors (SSRIs) have been investigated for the treatment of IFN- α induced depression. For instance, sertraline showed to be efficient in resolving depression without interruption of the IFN- α therapy (Schramm, Lawford *et al.* 2000). Similar results were obtained with other SSRIs, such as paroxetine (Musselman, Lawson *et al.* 2001, Kraus, Schafer *et al.* 2002, Raison, Woolwine *et al.* 2007) and escitalopram (Schaefer, Sarkar *et al.* 2012, Qi, Zhou *et al.* 2013), used both as a preventive measure and as a therapy for depression. In our study, five patients received antidepressants during the study period which could explain the decline in depression scores in the second semester of the treatment. However, changes in depressive symptoms over time were not different between patients that received antidepressant treatment and those that did not, as there was no significant interaction between antidepressant treatment and time on the depression scores. This conclusion should however be taken with caution since the number of

152

patients may be too low to find differential changes in depressive scores between both groups of patients.

A prominent role of the KYN pathway in major depression has been previously proposed (Maes, Mihaylova et al. 2007, Myint, Kim et al. 2007, Miura, Ozaki et al. 2008). Various studies reported decreased serum TRP (Maes, Verkerk et al. 1997), 5-HIAA (the main metabolite of serotonin) (Myint, Bondy et al. 2013), KYNA in plasma (Myint, Kim et al. 2007) and reduced ratio of KYNA/QUIN (Savitz, Drevets et al. 2015), but increased levels of KYN (Myint, Bondy et al. 2013) in some patients suffering from depression compared to controls. One group observed reduced TRP levels, but were unable to show changes in KYN, IDO, and other metabolites and enzymes of the KYN pathway (Hughes, Carballedo et al. 2012). Interestingly, a recent study showed a correlation between specific depressive symptoms and KYN pathway metabolites. For instance, QUIN concentration was positively correlated with the severity of guilt and the QUIN/3HK ratio was positively correlated with the severity of guilt and psychomotor agitation (Halaris, Myint et al. 2015).

It is well known that pro-inflammatory cytokines stimulate the KYN pathway (Chiarugi, Calvani et al. 2001, Guillemin, Kerr et al. 2001). Specifically, IFN- α and other pro-inflammatory cytokines activate IDO, the enzyme that converts TRP to KYN (Pemberton, Kerr et al. 1997, Lood, Tydén et al. 2015). Therefore, activation of the KYN pathway could be especially involved in IFN- α induced depression. In IFN- α treated patients, various studies reported decreased serum levels of TRP and serotonin, and increased KYN levels (Bonaccorso, Marino et al. 2002, Wichers, Koek et al. 2004, Cozzi, Zignego et

al. 2006). One recent study on the other hand showed increased TRP concentrations in the serum of patients treated with IFN- α (Oxenkrug, Turski et al. 2014), which may, although speculative, reflect an impaired transport of TRP into the brain. We did not find alterations in TRP during IFN- α treatment, but KYN levels steadily increased. Following the hypothesis of the activation of the KYN pathway, an increased KYN/TRP ratio, indicative of enhanced IDO activity, and a decreased KYNA/KYN ratio, indicative of augmented neurotoxic activity of the KYN cascade, are observed in IFN- α treated patients (Wichers, Koek et al. 2004). Moreover the KYNA/KYN ratio was negatively associated over time with MADRS score (Wichers, Koek et al. 2004). Another study reported decreased concentration of serum total TRP during IFN- α treatment, and increased KYN levels during treatment with IFN- α (Comai, Cavalletto et al. 2011). The results of both studies are in agreement with those reported here. We as well observe increases in KYN and the KYN/TRP ratio, and a reduction in the KYNA/KYN ratio during IFN- α treatment, indicative of enhanced IDO activity and reduced production of the neuroprotective KYNA, respectively. We also found that the KYNA/KYN ratio was negatively associated over time with the MADRS score. To our knowledge, this study is the first that reports peripheral measurements of other metabolites, e.g. increased QUIN and 3HK over time in IFN- α treated patients. One study showed increased 3HAA levels after 8 weeks of IFN- α therapy. As 3HAA is the direct precursor of QUIN, they considered it as an index for neurotoxic burden of metabolites of the KYN pathway (Van Gool, Verkerk et al. 2008). To sum up, evidence points to an activation of the KYN pathway during IFN- α therapy, specifically of the neurotoxic arm of the

154

pathway. Our study, with the most extensive analysis of the metabolites of the KYN pathway, further supports this theory, showing that IFN- α increases the neurotoxic metabolites QUIN, KYN and 3HK, and that development of depressive symptoms is negatively associated with the neuroprotective KYNA metabolite.

Several studies have used the KYN/TRP ratio as an indicator of IDO-activity and mainly reported increases during IFN- α therapy (Bonaccorso, Marino et al. 2002, Capuron, Neurauter et al. 2003, Wichers, Koek et al. 2004, Comai, Cavalletto et al. 2011). One study did not detect such changes (Oxenkrug, Turski et al. 2014). No studies so far have directly examined the gene expression levels of the enzymatic players in the KYN pathway during IFN- α treatment. We show an increase in KMO and KYNase over time during IFN- α treatment, indicative of the increased synthesis of neurotoxic 3HK and 3HAA. In addition, KYNase expression significantly predicted 3HK levels. On the other hand, during IFN- α treatment, gene expression levels of KAT-3 were increased, but not the levels of its product KYNA. The increase of KAT-3 levels may be explained because of the increase of its substrate KYN. However, the fact that no changes are observed in KYNA, suggests an activation of the neurotoxic arm of the KYN pathway over the neuroprotective one. Taken together, these data indicate that enhanced KYN pathway activity during IFN- α treatment is reflected in increased expression of enzymes related to the production of neurotoxic KYN metabolites.

As we already mentioned, some patients received antidepressant treatment during the study. It may be possible that antidepressants produce some changes in metabolites or enzymes of the KYN pathway. A recent paper analyzed the effects of antidepressant treatment in several metabolites and ratios of the KYN pathway in depressed patients. They observed reductions of the neurotoxic 3HK, QUIN and a decrease in 3HK/KYN and QUIN/TRP ratios. On the other hand, the KYNA/QUIN ratio was increased (Halaris, Myint et al. 2015). In our study, all analyses were corrected for the use of antidepressants, benzodiazepines and opioids. Regression coefficients, nor significance levels, were changed when adding these variables to the models, indicating that use of antidepressants during the study period had no major effect on our analysis. Moreover, it is interesting to note that while depressive symptoms decrease during the second part of the study period, the KYN/TRP ratio – and to a lesser extent KYN, 3HK and QUIN levels – steadily increase throughout. It is tempting to speculate that the neurotoxic metabolites induced by IFN- α initially increase depression scores, but that the subsequent relief of depressive symptoms after extended IFN- α exposure, whether are not mediated by antidepressants, is mediated by compensatory mechanisms that are unrelated to this part of the KYN pathway. Clearly, further examination of these processes could shed new light on the role of the KYN pathway in major depression and in IFN- α induced depression.

Despite the small sample size, this study shows a role for the KYN pathway in the development of depression after 48 weeks of treatment with IFN- α , as indicated by the increasing KYN/TRP ratio over time. Specifically, we

report enhanced levels over time of the considered neurotoxic metabolites of the KYN pathway, as well as increased concentrations of the enzymes regulating this arm of the pathway. In addition, the negative association observed between the ratio KYNA/KYN and MADRS scores, indicates the importance of the neuroprotective KYNA in the development of depressive symptoms. Evidently, it would be of great value to further explore the exact role of the KYN pathway in IFN- α induced depression in order to understand the precise mechanisms involved. Further research should also address how antidepressants, as a preventive measure or therapy, could affect the above mentioned metabolites and enzymes.

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Chapter 7

General discussion

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General discussion

Increasing evidence shows that infection during the pregnancy affects brain development in the offspring, increasing their susceptibility to develop psychopathology later in life. The underlying mechanisms mediating this relation still remain to be elucidated though. Several mediators have been proposed to be involved, such as epigenetic mechanisms and the kynurenine metabolism. The possible role of these factors is examined in the different chapters of this thesis.

Chapter 2 represents a review of the literature, addressing the association between prenatal maternal infection and the development of psychiatric disorders in the offspring in later life, in particular depression. Several hypotheses on the pathogenesis of depression and the role of possible mediators, such as epigenetic programming, are being discussed. In addition, we addressed the function of the immune system during normal pregnancy and the changes observed after infection during gestation. Moreover, the kynurenine pathway and downstream neurotoxic and neuroprotective metabolites are discussed. In addition, this chapter highlights the consequences of pregnancy stress on the immune system, the effects of which are strikingly similar to those of prenatal maternal infection. As depression is more likely to occur in women, gender differences are also evaluated. Lastly, an overview of the current available treatment options is given.

While reviewing the literature, we could notice a lack of direct evidence linking prenatal maternal infection and the development of depression-related phenotypes in the offspring, both in animal and human studies. In

animals, exposure to prenatal infection has been mostly studied in relation to the development of schizophrenia (Zuckerman, Rehavi et al. 2003, Meyer, Feldon et al. 2005, Zuckerman and Weiner 2005, Piontkewitz, Assaf et al. 2009, Bitanirwe, Peleg-Raibstein et al. 2010, Howland, Cazakoff et al. 2012, Wischhof, Irsack et al. 2015). In line with this, several epidemiological studies showed that infection during pregnancy was associated with an increased risk of schizophrenia in the offspring (Brown, Begg et al. 2004, Babulas, Factor-Litvak et al. 2006, Mortensen, Nørgaard-Pedersen et al. 2007, Sørensen, Mortensen et al. 2009). Interestingly, we were not able to find similar studies related to the development of depression. Moreover, only few animal studies examined the consequences of prenatal maternal infection on depression-related behavior (Enayati, Solati et al. 2012, Khan, Fernando et al. 2014). Clearly, it would be of great interest to further explore the possible link between prenatal infection and the development of depression in the offspring employing both animal models and epidemiological studies. Thus, prospective human studies together with more epidemiological studies exploring the above mentioned link would be of great value in the field.

In Chapter 3, we examined how a subcutaneous injection of LPS at GD 17 influenced gestational length and offspring mortality. It is known that the effects of infection exerted on the dam and the offspring differ depending on the timing, dose and route of administration (Mijovic, Zakar et al. 2002, Nakamura, Mori et al. 2003, Renaud, Cotechini et al. 2011). As such, it is challenging to find the right combination of dose, route and timing of injection that will produce the effects that the investigator is aiming for.

Moreover, it is remarkable that pregnant rodent dams are commonly subjected to an intraperitoneal injection (Buhimschi, Buhimschi et al. 2003, Xu, Chen et al. 2006, Salminen, Paananen et al. 2008), as a misplaced injection could be dangerous for both the mother and the foetus(es). Given the lack of evidence using subcutaneous injections of LPS in pregnant mice, we decided to test the effects of subcutaneous LPS administration using a dose of 2 mg/kg and 0.5 mg/kg. We found that gestational length was markedly reduced and neonatal mortality was dramatically increased after using the higher dose of LPS. In addition, litter size tended to be reduced after the administration of a low dose of LPS. More specifically, 2 mg/kg of LPS injected subcutaneously resulted in an abrupt *premature* delivery, concomitant with an elevated degree of *neonatal* mortality, while injection of 0.5 mg/kg LPS caused an increase in *prenatal* mortality that could be explained by reabsorption of foetuses. Altogether, we suggest that subcutaneous injections may be more suitable than intraperitoneal injections when dealing with pregnant dams. Following the studies performed here, it would be of clear added value to examine the exact effects of maternal LPS exposure in pups when using different doses, routes and time points of administration. This will help to choose the suitable combination when planning future experiments.

In Chapter 4 we investigated brain quinolinic acid (QUIN) expression in naïve adult mice. Altered QUIN expression has been reported in patients suffering from psychiatric disorders (Németh, Toldi et al. 2006, Gulaj, Pawlak et al. 2010, Raison, Dantzer et al. 2010), while its neurotoxic properties are well known (Nemeth, Toldi et al. 2005, Maes, Yirmiya et al. 2009). Although

several studies had already examined QUIN expression in different disease states and models (Baratte, Molinari et al. 1998, Guillemín, Brew et al. 2005, Guillemín, Smythe et al. 2005, Chung, Leung et al. 2009), knowledge on its distribution and pattern of staining in naïve mice was still lacking. We showed that QUIN expression was particularly pronounced in the cingulate cortex (CC) and the thalamic reticular nucleus (TRN) and to a similar degree in males and females. In the CC, we could observe clearly delineated individual cells, whereas in the TRN the staining pattern was much more diffuse. Furthermore, we found that the number of QUIN-positive cells observed in the CC was correlated to the degree of QUIN-immunoreactivity in the TRN. Interestingly, these two brain areas are functionally and anatomically connected and it is known that the TRN receives projections from the cortex (Pinault 2004, Çavdar, Onat et al. 2008). In addition, in males only, QUIN-immunoreactivity in the TRN was strongly correlated with locomotion. This association suggests QUIN as a possible mediator in regulating motor behavior at the level of the TRN. Interestingly, the TRN has been reported to be involved in the attention deficit hyperactivity disorder (ADHD), specifically in the modulation of arousal (di Michele, Prichep et al. 2005, Rowe, Robinson et al. 2005), and a recent paper showed a positive correlation between the baseline QUIN/3HK ratio and the severity of psychomotor agitation in patients with major depression (Halaris, Myint et al. 2015). It would be interesting in future studies to perform behavioural tests more closely related to primary functions of the CC and the TRN, such as memory and navigation. Furthermore, it would be of value to examine the exact nature of the cells displaying QUIN-immunoreactivity, e.g. by using

double-labeling immunofluorescence. Moreover, our findings, in line with recent evidence from other studies suggest that the role of QUIN in locomotion should be further investigated as well as the role of QUIN in the CC and the TRN in models of stress and/or inflammation linked to depression. Finally, the evaluation of other metabolites such as kynurenic acid, may shed new light on the field.

In Chapter 5, we evaluated changes in global enzymatic histone deacetylase (HDAC) and DNA methylation (DNMT) activity, after prenatal maternal exposure to Poly I:C at GD17. Poly I:C has been commonly used to mimic a viral infection (Doukas, Cutler et al. 1994, Snell, Chernyshev et al. 1997). Infection and consequent inflammation during gestation have been associated with an increased risk to develop psychiatric disorders in the offspring in later life (Fatemi, Reutiman et al. 2008, Zhou 2012). In this study, we investigated the possible role of epigenetic changes, i.e. in HDAC and DNMT activity, as potential mechanisms underlying the association between infection during pregnancy and offspring psychopathology. Results indicated a sex-specific effect of prenatal maternal Poly I:C exposure on brain HDAC activity, with an increased activity observed in female - but not male - Poly I:C offspring when compared to controls. Subsequently, we speculated which HDAC isoform may be mediating the increased activity observed. In this respect, western blot experiments could, however, not identify a specific HDAC. We examined HDAC1, HDAC2, HDAC3, HDAC4 and HDAC6, because of the evidence showing their association with mental (dys)function (McQuown, Barrett et al. 2011, Bahari-Javan, Maddalena et al. 2012, Fukada, Hanai et al. 2012, Morris, Mahgoub et al. 2013, Sarkar, Chachra et al. 2014).

Furthermore, we initially included HDAC5, because of its role in inflammation (Cantley and Haynes 2013), and that reduced levels of HDAC5 have been associated to the antidepressant effects of imipramine in mice models (Tsankova, Berton et al. 2006). However, we were not able to identify a specific band for HDAC5 after using various antibodies in several organ tissues. Possibly, other HDAC molecules than those assessed may be involved in mediating the effects of prenatal maternal Poly I:C exposure on global HDAC activity. In addition, a higher enzymatic activity does not necessarily imply an increase in the level of the associated protein(s). Clearly, this issue awaits further research. Of note, as mentioned in Chapter 5, it would of additional interest to examine the brain region- and cell-type-specific epigenetic effects of prenatal maternal Poly I:C exposure in order to unravel the exact role of epigenetic programming in mediating the effects of prenatal maternal infection. In addition to differences in the degree of histone acetylation, in part mediated via altered HDAC activity, it would be of great value to identify other epigenetic players that mediate the effects of prenatal maternal infection on offspring brain development.

In Chapter 6, we explored the role of the kynurenine pathway in relation to the development of depressive symptoms in patients treated with interferon-alpha (IFN- α). Patients diagnosed with hepatitis C are commonly treated with IFN- α , and a considerable percentage has been reported to develop depression (Papafragkakis, Rao et al. 2012). It is well-known, that cytokines, such as IFN- α , regulate diverse enzymes of the kynurenine (KYN) pathway (Myint 2012), whose metabolites have been found altered in depressed patients (Bonaccorso, Marino et al. 2002, Capuron, Neurauter et

al. 2003, Wichers, Koek et al. 2005, Cozzi, Zignego et al. 2006, Comai, Cavalletto et al. 2011, Hughes, Carballado et al. 2012, Myint, Bondy et al. 2013, Oxenkrug, Turski et al. 2014), albeit with discordant results. Thus, we decided to analyse gene expression levels of several enzymes as well as concentrations of metabolites of the KYN pathway in IFN- α treated patients. We observed significant changes during the 48 weeks of IFN- α treatment in the Montgomery-Asberg Depression Rating Scale (MADRS) and the Beck Depression Inventory (BDI), Kynurenine (KYN), 3-Hydroxykynurenine (3HK), Quinolinic acid (QUIN), the ratio Kynurenine/Tryptophan and the Kynurenic Acid/Kynurenine ratio. In addition, kynureninase, kynurenine-3-monooxygenase and kynurenine aminotransferase 3 enzymes were changed over time during the IFN- α treatment. Next, we observed that only KYNA and the ratio KYNA/KYN were negatively associated with MADRS scores over time, whereas no association was found between gene expression levels of the studied enzymes and MADRS scores. Finally, we analysed possible associations between the enzymes and their precursors and products. Only 3HK was positively associated with KYNase. These results suggest that the KYN pathway, and specifically its neurotoxic metabolites, e.g. 3HK, QUIN and the enzymes that regulate the neurotoxic arm of the pathway, have a role in IFN- α induced depression. On the other hand, the neuroprotective arm, as represented by KYNA levels, is negatively associated with depressive symptoms. Overall, IFN- α shifts the neurotoxic/neuroprotective balance of the KYN pathway metabolites to the neurotoxic arm resulting in the precipitation of depressive symptoms. Future investigations should further examine the KYN pathway in order to better understand the exact

170

mechanisms involved in IFN- α induced depression and in general major depression. It would be also of interest to assess whether or not the therapeutic or prophylactic use of antidepressants may influence metabolites and enzymes of the KYN pathway.

Methodological considerations

All in all, the work associated with this thesis yielded numerous valuable insights regarding the design and execution of animal work on prenatal maternal infection, the most important of which concerns controlling for litter effects. Pups from a single litter show a limited degree of genetic, epigenetic and environmental variation (Meyer, Feldon et al. 2009). Thus, when assessing the effects of prenatal events on measures in the offspring, we may confound treatment effects with so-called litter effects (Chapman and Stern 1978, Lazic and Essioux 2013). Principally, in studies like these, the dams/litter should be considered as the experimental/statistical unit, and not the pup(s). A way to avoid these litter effects is including only 1-2 pup(s) per sex from each litter when assessing a specific experimental variable (Hass, Ladefoged et al. 2001, Upton and Sullivan 2010). In Chapters 3 and 4, pregnant dams were exposed to maternal infection. Mice were injected s.c. or i.p. with LPS, Poly I:C or PBS, respectively, at GD17. After weaning, offspring was separated by sex and treatment, whereas litter effects were not controlled for. Unfortunately, at a later stage, it was impossible to trace back which pups belonged to the same litter. Consequently, the behavioural measurements assessed in these animals could not be used to directly compare offspring exposed to prenatal maternal LPS or Poly I:C and controls.

Concluding remarks

Psychiatric disorders are leading cause of disability worldwide. More specifically, major depression affects more than 100 million people per year. Despite the repercussion of this disorder on our personal life, economy and health, a reliable biomarker to diagnose depression has not been developed yet, while treatment efficacy is relatively low. In this thesis, we tried to assess a variety of factors, such as epigenetic processes as well as metabolites and enzymes of the kynurenine pathway, in the context of inflammation, in order to identify potential biomarkers and an effective treatment for depression. We observed that LPS administration during pregnancy in the mouse had a profound impact upon gestational length, litter size and perinatal mortality in the offspring, even at a relatively low dose. In the long run, female offspring of dams exposed to Poly I:C during pregnancy showed increased global HDAC activity when compared to control offspring. Regarding the role of the kynurenine pathway, we observed QUIN-immunoreactivity in two brain areas in naïve mouse, the CC and the TRN. Interestingly, we observed an association between QUIN-immunoreactivity in the TRN and locomotion, which suggests that QUIN is involved in the regulation of motor activity. Furthermore, the role of kynurenine pathway was assessed in IFN- α treated patients, indicating the importance of the neurotoxic metabolites and related enzymes of the pathway, and the association between depressive symptoms and reduced neuroprotective metabolites.

We hypothesized that alterations in the kynurenine pathway are causally involved in mediating changes in inflammation-induced depression-related

phenotypes. Moreover, we postulated epigenetic changes as mechanisms underlying the association between prenatal inflammation and adult psychopathology. Altogether, we showed that prenatal maternal inflammation mediated changes in epigenetic marks, while exposure to cytokines in adulthood affected the kynurenine metabolism in relation to depression. A better understanding of the processes underlying the negative consequences of prenatal maternal inflammation as well as those induced by exposure to cytokines in adulthood will help for an early diagnose and better targeted treatment.

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Summary

In this dissertation, we studied the role of inflammation in depression. Concretely, epigenetic changes after prenatal inflammation and the role of the kynurenine pathway in depression were investigated.

First, **Chapter 1** provides an overview of the background of the disease, the current tools used for diagnosis and treatment, and the different pathophysiological theories related to depression. Moreover, the role of epigenetic mechanisms in the link between prenatal infection and depression in the offspring are addressed.

Chapter 2 represents a review about the current literature on how exposure to prenatal maternal infection may interfere with offspring brain development. More specifically, the immune system and the possible involvement of several mediators such as cytokines, neurotransmitters, neurotrophins, hormones and epigenetic processes are reviewed. Moreover, the effects of prenatal maternal stress and infection in the offspring, in view of inflammation, are discussed. Finally, the chapter ends with an overview of sex-differences in response to prenatal maternal infection as well as treatment options for major depression.

Chapter 3 describes the effects of lipopolysaccharide (LPS), an endotoxin contained in bacterial cell walls, on gestational length and intra-uterine and neonatal mortality in mice. The induction of prenatal inflammation using a high dose of LPS significantly decreased gestational length and increased neonatal mortality. When using a low dose of LPS, we observed a tendency

towards reduced litter size. In addition, we discussed the advantages of using subcutaneous injections instead of intraperitoneal injections, when dealing with pregnant animals.

Chapter 4 analyses quinolinic acid (QUIN) in the naïve mouse brain in relation to affect-related behavior. We focused on the localization of QUIN in the naïve mouse because of the lack of knowledge regarding QUIN in healthy, control conditions. We showed QUIN-immunoreactivity particularly in the cingulate cortex (CC) delineating individual cells and in the thalamic reticular nucleus (TRN) resembling a fibrillary staining pattern. Moreover, we observed a strong correlation between QUIN-immunoreactivity in the TRN and motor activity in the open field test, suggesting a role of QUIN in the regulation of motor activity in normal conditions.

In **Chapter 5** we assessed alterations in epigenetic mechanisms in the mouse brain after prenatal exposure to polyinosinic:polycytidylic acid (Poly I:C). More specifically, we examined global histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity within the brain. We found a significant sex x treatment interaction effect after prenatal exposure to maternal immune challenge by Poly I:C, indicative of increased global HDAC activity particularly in female offspring from mothers injected with Poly I:C when compared to controls. In addition, the levels of the isoforms HDAC 1,2,3,4 and 6 were explored as a possible explanation for the increase observed in global HDAC activity. However, we could not detect significant higher levels of the HDAC isoforms examined in Poly I:C versus control female offspring.

Although we observed a treatment effect with increased HDAC6 in offspring of mice treated with Poly I:C compared to controls, this effect seemed to be more pronounced in male - and not female - offspring, which does not match the global HDAC activity data. A feasible explanation for the discrepancy observed between the global HDAC activity and the protein levels of several HDAC isoforms in the present study is that one or more other HDAC isoform(s) than those assessed using western blotting is/are involved in mediating the observed increased in global HDAC activity. Taken together, this study indicates that prenatal maternal Poly I:C exposure seems to increase global HDAC, but not DNMT activity, particularly in female offspring. Altogether, though purely speculative, these data suggests a role for HDAC6 in mediating the association between prenatal maternal infection and the development of depression in the offspring.

Chapter 6 investigates alterations in the depression scores and in diverse enzymes and metabolites of the kynurenine pathway in Interferon-alpha (IFN- α) treated patients. We report a significant change over time in both the Montgomery-Asberg Depression Rating Scale (MADRS) and the Beck Depression Inventory (BDI) scores during IFN- α therapy. In addition, levels of the neurotoxic metabolite kynurenine (KYN), 3-hydroxykynurenine (3HK), quinolinic acid (QUIN) and the kynurenine/tryptophan (KYN/TRP) ratio were increased over time, while the kynurenic acid/kynurenine (KYNA/KYN) ratio, reflecting neuroprotective properties, was reduced. Regarding the enzymes of the kynurenine pathway, the activity of kynureninase (KYNase), kynurenine-3-monooxygenase (KMO) and kynurenine aminotransferase

(KAT-3) were also increased. When examining the relationship between MADRS scores and metabolites, KYNA and the KYNA/KYN ratio were negatively associated with MADRS scores. On the other hand, no association was observed between enzymes and the MADRS score. Finally, relative expression of KYNase significantly predicted levels of 3HK. Altogether, our results confirm, that the kynurenine pathway plays an important role in the development of depressive symptoms during IFN- α treatment, with a shift in the balance between neurotoxic and neuroprotective metabolites, as well as increased expression of enzymes regulating the neurotoxic arm of the pathway.

Finally, in **Chapter 7**, a summary and discussion of the main findings of this thesis are provided. In addition, some methodological considerations are addressed. Finally, concluding remarks are disclosed.

To sum up, in this thesis, we investigated possible mediators of the effects produced during brain development in offspring after prenatal inflammation, such as epigenetic mechanisms. In addition, we explored the role of QUIN in healthy conditions. Finally, we examined the role of several enzymes and metabolites of the kynurenine pathway in relation to depression in IFN- α treated patients. To conclude, we showed that prenatal maternal inflammation mediated changes in epigenetic marks, while exposure to cytokines in adulthood affected the kynurenine metabolism in relation to depression.

Valorization

Psychiatric disorders have a large impact on our society. More than 350 million people are estimated to suffer from depression including an increasing number of young persons. Concretely, major depression is the leading cause of disability with a high social, economic and societal burden (WHO 2012). Patients suffering from depression show a reduced capacity to study or work, which considerably affects the economy (Gilbody, Bower et al. 2012, McTernan, Dollard et al. 2013). Moreover, suicide and divorce rates are increased in depressed patients (Wolfersdorf 2008).

Major depression is a heterogeneous and complex disorder compromising a wide spectrum of symptoms, such as low mood, insomnia, fatigue and anhedonia among others (Kennedy 2008). There still is a lack of knowledge of the underlying cause of depression, which dramatically complicates its diagnosis and treatment. Diagnosis is still based on a clinical psychiatric interview, due to the lack of suitable biomarkers. Treatment consists mainly of antidepressants, which increase the concentration of monoamine neurotransmitters in the synaptic cleft. However, it is still unclear whether the therapeutic effect of antidepressants is related to their monoaminergic actions. For instance, while antidepressants rapidly increase monoamine levels, weeks or months of treatment are required before clinical improvement is observed. In addition, treatment efficiency is only 60-65%, with a remission rate of around 30% (Block and Nemeroff 2014), and at least 20% of depressed patients are treatment-resistant (Berlim and Turecki 2007). In view of the high societal burden, it is vital to deepen our understanding of the biological mechanisms of depression in order to unveil new potential therapeutic targets, such as the kynurenine pathway.

Depression has been linked to inflammation, since a subgroup of depressed patients show higher circulating levels of pro-inflammatory cytokines (amongst other increased inflammatory markers), and major depression is frequently comorbid with many inflammatory diseases (Anisman and Hayley 2012, see review by Gray and Bloch 2012). Interestingly, cytokines regulate several enzymes of the kynurenine pathway (Myint 2012), leading to the production of several neurotoxic, e.g. quinolinic acid (QUIN), metabolites. Thus, the kynurenine pathway has been considered as a possible mechanism involved in the pathophysiology of depression (Maes, Mihaylova et al. 2007, Myint, Kim et al. 2007, Miura, Ozaki et al. 2008) and its metabolites and enzymes may serve as potential biomarkers (Fukuda 2014).

Many studies have examined the levels of different metabolites of the kynurenine pathway in depressed patients (Sublette, Galfalvy et al. 2011, Ogawa, Fujii et al. 2014, Savitz, Drevets et al. 2015). However, the full range of metabolites in conjunction with the expression of genes involved in the kynurenine pathway has not been examined yet. In this thesis, we show increased levels of some considered neurotoxic metabolites of the pathway e.g. kynurenine (KYN), 3-hydroxykynurenine (3HK) and QUIN, as well as increased expression of the enzymes regulating this arm of the pathway. In addition, the negative association observed between the kynurenic acid/kynurenine (KYNA/KYN ratio and scores in the Montgomery-Asberg Depression Rating Scale (MADRS), underscores that development of depressive symptoms is mediated by a disturbance in neuroprotective support. Altogether, we found altered levels of neurotoxic and

neuroprotective metabolites in IFN- α treated patients, consolidating the possible use of these metabolites as biomarkers for depression.

In particular, QUIN has been studied extensively, because of its known neurotoxic effects (Nemeth, Toldi et al. 2005). Although physiological levels of QUIN are necessary in healthy conditions to synthesize nicotinamide adenine dinucleotide (NAD), which is needed for any cell to function properly (Myint 2012), evidence is lacking regarding QUIN in normal physiological conditions. Therefore, in Chapter 4 we investigated in the brain of naïve mice the localization of QUIN, the pattern of its staining, and its possible association with a subset of affect-related behavioural phenotypes. We detected QUIN-immunoreactivity, particularly in the cingulate cortex and the thalamic reticular nucleus (TRN), and it was strongly correlated with locomotor activity in males. This suggests an important role for QUIN at the level of the TRN in regulating motor behaviour. Interestingly, the TRN has been involved in attention-deficit hyperactivity disorder (ADHD), specifically in the modulation of arousal (di Michele, Prichep et al. 2005, Rowe, Robinson et al. 2005). In addition, recently a study reported that the QUIN/3HK ratio was positively correlated with psychomotor agitation in depressed subjects (Halaris, Myint et al. 2015). To sum up, we show that QUIN is observed in normal conditions in the brain and that it may play a role in the regulation of motor behavior. Thus, the conception of QUIN just as a neurotoxic factor should be further clarified.

Another mechanism that has been associated with depression is epigenetic programming. Specifically, changes in histone acetylation have been involved in the pathophysiology of depression (Tsankova, Berton et al.

2006). In addition, histone deacetylase (HDAC) inhibitors have been reported to exert antidepressant effects (Yamawaki, Fuchikami et al. 2012, Hsing, Hung et al. 2015) and have been suggested as possible targets for the future development of novel antidepressant treatments (Covington, Maze et al. 2009). Therefore, we studied changes in epigenetic processes in a maternal immune activation mouse model for depression using a viral mimetic, e.g. polyinosinic:polycytidylic acid (Poly I:C). We observed an increase in global HDAC enzymatic activity, particularly in females' offspring exposed prenatally to Poly I:C. Further investigations on the specific HDAC isoforms involved and the effects produced by HDAC inhibitors, would be of great value.

To conclude, this thesis aimed to provide more evidence on the current approaches to diagnose and treat depression. Our results are certainly of interest to subjects undergoing IFN- α or pro-inflammatory cytokine therapy and to depressed patients suffering from an inflammatory condition. To our knowledge, we are the first to analyse the complete KYN pathway in conjunction with the gene expression levels of the enzymes involved, in IFN- α induced depression. Besides, we combined the measurements of global DNA methyltransferase (DNMT) and HDAC activity with specific HDAC isoform measures. Our studies support further investigation in the use of HDAC inhibitors as new targets for the treatment of depression. In addition, we reaffirmed the use of metabolites of the kynurenine pathway, e.g. KYN, 3HK and QUIN and the enzymes regulating this neurotoxic arm, kynureninase (KYNase) and kynurenine-3-monooxygenase (KMO), as

potential biomarkers for the diagnosis of depression. These metabolites and enzymes mentioned above could also be used to differentiate depressed patients with or without underlying inflammatory conditions. In addition, their measurement before and after treatment, might help in the prediction of the therapeutic response.

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Mis amigas de la Autónoma. Mis biólogas: Alba, Cris y Vero, que deciros después de tantos años de amistad (12 ya madre mía!!!!). Nunca olvidare todas las aventuras que hemos vivido juntas, desde las excursiones de botánica, partidas de cartas en la cafetería, nuestras comidas en el mandri, las noches de fiesta en Barcelona, los viajes que hemos hecho juntas, y que no han sido pocos jajaja! Os estoy muy agradecida de vuestra amistad, nunca pensé que pudiera conseguir tan grandes amigas en la universidad y lo mejor es que sé que todavía nos quedan muchas cosas juntas por vivir.

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Y aquí reservo un espacio para Verónica también. Te conocí un poco más tarde en San Francisco, pero eres como una compi más de la universidad. Muchas gracias por todos esos descuentos en super hoteles y por todos esos viajes geniales donde tanto hemos conocido y disfrutado y todos los que nos quedan por hacer! También por tus visitas a Múnich y Maastricht! Próximo destino?????

Y también Andrea! Tu eres la encargada de cerrar el círculo de PhD! Muchas gracias también por acogerme siempre y por todas esas fiestas y charlas! Nos vemos en tu defensa!!!!

També a tu Carol. Mai oblidaré la oportunitat que hem vau donar de fer les meves primeres pràctiques, de trepitjar per primera vegada un laboratori real, de tot el que em vas ensenyar. Sé que vas treballar molt dur per aconseguir el treball dels teus somnis, i admiro que encara ara després de tants anys no hakis perdut la il·lusió i sapiguis contagiar-la als teus estudiants. I lo millor es que encara poguem fer un Viena de tant en tant després de tants anys i posar-nos al dia! T'estaré sempre agraïda!!!

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Oktoberfest, bueno todo lo que acabe en fest jajajaja, bodas, viajes, domingos santos de películas y charlas, sobretodo nuestras charlas. Sabes que siempre puedes visitarnos en Reus (in-de, independencia jajajaja) y a mi allá donde este. Queda pendiente nuestro viaje a México!!!

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Zuri: juntas empezamos nuestra aventura en Múnich y descubrimos esa ciudad. Estuviste conmigo desde el principio, todavía recuerdo aquella lista de qué hacer para encontrar mi trabajo que hicimos en un Biergarten jajaja! Gracias por tu apoyo siempre y por todos los momentos que hemos disfrutado juntas, desde vacaciones como olvidar Croacia, en Reus, y los días en Maastricht, cenas, wiesn, cervezas, fiestas, mudanzas, cafés, charlas y todo lo que nos queda. Eskerrik asko!!!

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Curriculum vitae

Yara Pujol López was born on the 25th of March 1985 in Reus, Spain. She obtained her High School degree with specialization in Health Sciences at the Col·legi Sant Josep (Reus, Spain) in 2003. Next, she moved to Barcelona and obtained her bachelor's degree in Biology in 2008 with specialization in Sanitary Biology at the Universitat Autònoma de Barcelona (Barcelona, Spain). In her hometown, she conducted her final internship in the Center of Human Reproduction Biogest, (Reus, Spain) under the supervision of Carolina Matas. Immediately afterwards, she started her Master in Human Reproduction in the Universidad Complutense de Madrid (Madrid, Spain), conducting her final internship in the Center of Infertility and Human Reproduction (CIRH, Barcelona, Spain). Her next step took her away from the academic world and moved to San Francisco, USA to improve her English. Yara then rejoined the academic world and moved to Munich, Germany. In 2011 she began her PhD at the Clinic of Psychiatry and Psychotherapy at the Ludwig-Maximilians-University (LMU) under the supervision of Dr. Aye-Mu Myint. In 2013, she moved to Maastricht, The Netherlands to continue her PhD at the School of Mental Health and Neurosciences, Maastricht University under the supervision of Prof. Harry Steinbusch, Dr. Daniel van den Hove and Dr. Gunter Kenis. In her PhD project she investigated the developmental and psychoneuroimmunological mechanisms in depression. The results of this work are described on this thesis.

Publication list

Peer-reviewed publications

* **Pujol Lopez Y**, Steinbusch HWM, Rutten BPF, Kenis G, van den Hove D, Myint AM.

Effects of subcutaneous lipopolysaccharide (LPS) injection on gestational length and intrauterine and neonatal mortality in mice.
Neuroimmunomodulation 2015;22(4):274-8

Submitted / under review manuscripts

* **Pujol Lopez Y**, Kenis G, Stettinger W, Neumeier K, de Jonge S, Steinbusch HWM, Zill P, van den Hove D, Myint AM.

Effects of prenatal polyinosinic:polycytidylic acid (Poly I:C) exposure on global histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activity in the mouse brain.

* **Pujol Lopez Y**, Kenis G, Rutten BPF, Myint AM, Steinbusch HWM, van den Hove D.

Quinolinic acid-immunoreactivity in the naïve mouse brain.

Manuscripts in preparation

* **Pujol Lopez Y**, van den Hove D, Steinbusch HWM, Kenis G¹, Myint AM¹.

Prenatal maternal infection and offspring brain development: programming of adult psychopathology (Review).

¹Authors contributed equally to this work

* **Pujol Lopez Y**, Leue C, Koek G, Myint AM, van den Hove D, Pishva E, Schwarz MJ, van Os J, Steinbusch HWM, Rutten BPF, Kenis G.

Depressive symptoms during IFN-alpha therapy in humans associated with changes in the kynurenine pathway.

Thesis defenses from MHeNs - School for Mental Health and Neuroscience

2013

Rob Havermans: **Bipolar disorder in daily life; Mood and cortisol responses to naturally occurring events.** Supervisor: Prof.dr. M. de Vries; Co-Supervisor: Dr. N. Nicolson.

Véronique Moers-Hornikx: **Deep brain stimulation and the cerebellum.** Supervisors: Prof.dr. J. Vles / Prof.dr. Y. Temel; Co-Supervisor: Dr. G. Hoogland.

Nicole Veldhorst-Janssen: **Intranasal delivery of rapid acting drugs.** Supervisors: Prof.dr. M. Marcus / Prof.dr. C. Neef; Co-Supervisor: Dr. P.H. van der Kuy.

Stéphanie Knippenberg: **Vitamin D and Multiple Sclerosis: immunological and clinical outcome.** Supervisor: Prof.dr. J. Cohen-Tervaert; Co-Supervisors: Dr. J. Damoiseaux / Dr. Y. Bols.

Erik D. Gommer: **Dynamic Cerebral Autoregulation: from methodology towards clinical application.** Supervisors: Prof.dr. W.H. Mess / Prof.dr. R.B. Panerai, UK; Co-Supervisor: Dr.ir. J.P.H. Reulen.

Olga A.H. Reneerkens: **Can PDE inhibition improve cognition? Translational insights.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. J. Prickaerts.

Lyzel S. Elias-Sonnenschein: **Clinical and biomarker correlates of genetic risk factors for Alzheimer's disease.** Supervisor: Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. P.J. Visser.

Diego F. Mastroeni: **Epigenetic Dysregulation and the Pathophysiology of Alzheimer's Disease.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. P.D. Coleman, Sun City, Arizona; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.

Leonidas Chouliaras: **Epigenetic Regulation in Aging and Alzheimer's disease: A translational perspective.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. B.P.F. Rutten / Dr. D.L.A. van den Hove.

Liesbeth Knaepen: **Perinatal events and altered pain sensitivity in later life.** Supervisors: Prof.dr. E.A.J. Joosten / Prof.dr. D. Tibboel, EUR; Co-Supervisor: Dr. J. Patijn.

Marisela Martinez-Claros: **Hippocampal plasticity and corticosterone: From dendrites to behaviour.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawluski / Dr. J. Prickaerts.

Marcus D. Lancé: **A circle of improvement in bleeding management: from laboratory to clinic and back.** Supervisors: Prof.dr. M.A.E. Marcu / Prof.dr. J.W.M. Heemskerk; Co-Supervisor: Dr. Y.M.C. Henskens.

Hilde Braakman: **Imaging the brain; neuronal correlates of cognitive impairment in children with frontal lobe epilepsy.** Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr. J.S.H. Vles; Co-Supervisors: Dr.ir. W.H. Backes / Dr. P.A.M. Hofman.

Willem H. van Zwam: **Aneurysmal subarachnoid hemorrhage: imaging strategies and cost-effectiveness aspects in diagnostic work-up and post-therapeutic follow-up.** Supervisors: Prof.dr. J.T. Wilmlink / Prof.dr. J.E. Wildberger; Co-Supervisor: Dr. P.A.M. Hofman.

Klara De Cort: **The Pathogenesis of Panic Disorder.** Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. E.J.L. Griez; Co-Supervisors: Dr. K.R.J. Schruers / Dr. I. Van Diest, Leuven.

Kim van Wijck: **Mind the Gap; experimental studies on splanchnic hyperfusion and gastrointestinal integrity loss in man.** Supervisors: Prof.dr. W.A. Buurman / Prof.dr. C.H.C. Dejong;

Co-Supervisor: Dr. K. Lenaerts.

Yvette Roke: **Antipsychotic-induced hyperprolactinemia in children and adolescents with mainly autism spectrum disorders. Prevalence, symptoms, clinical consequences and genetic risk factors.** Supervisors: Prof.dr. P.N. van Harten / Prof.dr. J.K. Buitelaar (RUN); Co-Supervisor: Dr. A. Boot (UMCG).

Fleur Goezinne: **Retinal detachment surgery: pre and postoperative prognostic factors.** Supervisors: Prof.dr. F. Hendrikse / Prof.dr. C.A.B. Webers; Co-Supervisor: Dr. E.C. La Heij (Amsterdam).

Ralph L.J.G. Maassen: **The Merits of Videolaryngoscopy during Glottic Visualisation for Endotracheal Intubation.** Supervisors: Prof.dr. M. Marcus / Prof.dr. A. van Zundert (University of Queensland).

Maria J. de Sousa Guerreiro: **The role of sensory modality in age-related distraction.** Supervisor: Prof.dr. C.M. van Heugten; Co-Supervisor: Dr. P.W.M. van Gerven.

Ine Rayen: **Effects of developmental fluoxetine exposure on neurobehavioral outcomes.** Supervisor: Prof.dr. H.W.M. Steinbusch; Co-Supervisors: Dr. J.L. Pawlusi / Dr. T.D. Charlier (Ohio University, USA).

Nynke M.G. Bodde: **Psychogenic non-epileptic seizures; a separate disorder or part of a continuum?** Supervisors: Prof.dr. R. van Oostenbrugge / Prof.dr. K. Vonck (UZ Gent); Co-Supervisors: Dr. R. Lazeron / Dr. A. de Louw (Epilepsiecentrum Kempenhaeghe, Heeze).

Alejandro M. Gomez: **Novel strategies for making myasthenia less gravis: targeting plasma cells and the neuromuscular junction.** Supervisor: Prof.dr. M.H. De Baets; Co-Supervisors: Dr. M. Losen / Dr. P. Martinez-Martinez.

Mohammad S. Rahnema'i: **Prostaglandins and Phosphodiesterases in the Urinary Bladder Wall.** Supervisors: Prof.dr. Ph. Van Kerrebroeck / Prof.dr. S. de Wachter (Universiteit Antwerpen); Co-Supervisor: Dr. G. van Koeveeringe.

Mariken B. de Koning: **Studying biomarkers in populations at genetic and clinical high risk for psychosis.** Supervisors: Prof.dr. T. Amelsvoort / Prof.dr. J. Booij (AMC).

Fabien Boule: **Epigenetic regulation of BDNF/TrkB signaling in the pathophysiology and treatment of mood disorders.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. L. Lanfumey (Universiteit Parijs); Co-Supervisors: Dr. D. van den Hove / Dr. G. Kenis.

2014

Iris Nowak-Maes: **Tinnitus; assessment of quality of life & cost-effectiveness.** Supervisors: Prof.dr. M. Peters / Prof.dr. B. Kremer; Co-Supervisors: Dr. M. Joore / Dr. L. Anteunis.

Marjolein Huijts: **Cognitive function in patients with cerebral small vessel disease.** Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. A.A. Duits / Dr. J. Staals.

Markus Gantert: **Fetal inflammatory injury as origin of long term disease: Lessons from animal models.** Supervisors: Prof.dr. B. Kramer / Prof.dr. L. Zimmermann; Co-Supervisor: Dr. A. Gavilanes.

Elke Kuypers: **Fetal development after antenatal exposures: Chorioamnionitis and maternal glucocorticoids.** Supervisors: Prof.dr. B.W. Kramer / Prof.dr. H.W. Steinbusch / Prof.dr. Suhas G. Kallapur (University of Cincinnati, Ohio, USA).

Pieter Kubben: **Ultra low-field strength intraoperative MRI for Glioblastoma Surgery.** Supervisor: Prof.dr. J.J. van Overbeeke; Co-Supervisor: Dr. H. van Santbrink.

Laura Baijens: **Surface electrical stimulation of the neck for oropharyngeal dysphagia in Parkinson's disease: therapeutic aspects and reliability of measurement.** Supervisor: Prof.dr. B. Kremer; Co-Supervisor: Dr. R. Speyer, Townsville.

Janneke Hoeijmakers: **Small fiber neuropathy and sodium channels; a paradigm shift.** Supervisor: Prof.dr. R.J. van Oostenbrugge; Co-Supervisors: Dr. C.G. Faber / Dr. I.S.J. Merkies.

- Stephanie Vos: **The Role of biomarkers in preclinical and prodromal Alzheimer's disease.** Supervisor: Prof.dr. F.R. Verhey; Co-Supervisor: Dr. P.J. Visser.
- Muriël Doors: **The Value of Optical Coherence Tomography in Anterior Segment Surgery.** Supervisors: Prof.dr. R.M. Nuijts / Prof.dr. C.A. Webers; Co-Supervisor: Dr. T.T.J.M. Berendschot.
- Anneke Maas: **Sleep problems in individuals with genetic disorders associated with intellectual disability.** Supervisors: Prof.dr. I. Curfs / Prof.dr. R. Didden.
- Sebastiaan van Gorp: **Translational research on spinal cord injury and cell-based therapies; a focus on pain and sensorimotor disturbances.** Supervisors: Prof.dr. B. Joosten / Prof.dr. M. van Kleef; Co-Supervisors: Dr. J. Patijn / Dr. R. Deumens, KU Leuven.
- Andrea Sannia: **High risk newborns and brain biochemical monitoring.** Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Alessandria, Italy / Dr. A.W.D. Gavilanes.
- Julie A.D.A. Dela Cruz: **Dopamine mechanisms in learning and memory: Evidence from rodent studies.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R.J. Bodnar, New York; Co-Supervisor: Dr. B.P.F. Rutten.
- René Besseling: **Brain wiring and neuronal dynamics; advances in MR imaging of focal epilepsy.** Supervisors: Prof.dr. A.P. Aldenkamp / Prof.dr. W.H. Backes; Co-Supervisor: Dr. J.F.A. Jansen.
- Maria Quint-Fens: **Long-term care after stroke; development and evaluation of a long-term intervention in primary care.** Supervisors: Prof.dr. J.F.M. Metsemakers / Prof.dr. C.M. van Heugten / Prof.dr. M. Limburg, Almere; Co-Supervisor: Dr. G.H.M.I. Beusmans.
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- Feikje Smeets: **The hallucinatory-delusional state: a crucial connection in the psychosis symptom network.** Supervisor: Prof.dr. J. van Os; Co-Supervisor: Dr. T. Lataster.
- Lies Clerx: **Alzheimer's disease through the MR-eye; novel diagnostic markers and the road to clinical implementation".** Supervisor: Prof.dr. F. Verhey; Co-Supervisors: Dr. P.J. Visser / P. Aalten.
- Sonny Tan: **The subthalamic nucleus in Parkinson's disease.** Supervisors: Prof.dr. Y. Temel / Prof.dr. H.W.M. Steinbusch / Prof.dr. T. Sharp, Oxford, UK / Prof.dr. V. Visser-Vandewalle, Koln.
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- Ron Handels: **Health technology assessment of diagnostic strategies for Alzheimer's disease.** Supervisors: Prof.dr. F.R.J. Verhey / Prof.dr. J.L. Severens (EUR); Co-Supervisor: Dr. M.A. Joore / Dr. C.A.G. Wolfs.
- Evelyn Peelen: **Regulatory T cells in the pathogenesis of Multiple Sclerosis: potential targets for vitamin D therapy.** Supervisors: Prof.dr. R.M.M. Hupperts / Prof.dr. J.W. Cohen Tervaert; Co-Supervisor: Dr. J.G.M.C. Damoiseaux / Dr. M.M.G.L. Thewissen, Diepenbeek.
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F. Brunner, Zürich / Dr. R. Perez, VUmc.

Dagmar Zeef: **An experimental model of Huntington's disease: Validation & Stimulation.** Supervisors: Prof.dr. Y. Temel / Prof.dr. H.W.M. Steinbusch; Co-supervisor: Dr. A. Jahanshahi.

Jeroen Decoster: **Breaking Down Schizophrenia into phenes, genes and environment.** Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. M. De Hert, KU Leuven; Co-Supervisor: Dr. R. van Winkel.

Eaja Anindya Sekhar Mukherjee: **Fetal Alcohol Spectrum Disorders: exploring prevention and management.** Supervisor: Prof.dr. L.M.G. Curfs; Co-Supervisor: Prof. S. Hollins, St. George's University of London, UK.

Catherine van Zelst: **Inside out; On stereotype awareness, childhood trauma and stigma in psychosis.** Supervisors: Prof.dr. Ph. Delespaul / Prof.dr. J. van Os.

Ibrahim Tolga Binbay: **Extended Psychosis Phenotype in the Wider Social Environment.** Supervisor: Prof.dr. J. van Os; Co-Supervisor: Dr. M. Drukker.

Frank Van Dael: **OCD matters in psychosis.** Supervisors: Prof.dr. J. van Os / Prof.dr. I. Myin-Germeys.

Pamela Kleikers: **NOXious oxidative stress: from head toe too and back.** Supervisors: Prof.dr. H.H.H.W. Schmidt / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. B. Janssen.

José Luis Gerardo Nava: **In vitro assay systems in the development of therapeutic interventions strategies for neuroprotection and repair.** Supervisors: Prof.dr.med. J. Weis / Prof.dr. H.W.M. Steinbusch; Co-Supervisor: Dr. G.A. Brook, RWTH Aachen.

Eva Bollen: **Cyclic nucleotide signaling and plasticity.** Supervisors: Prof.dr. H.W.M. Steinbusch / Prof.dr. R. D'Hooge, KU Leuven; Co-Supervisor: Dr. J. Prickaerts.

2015

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Bart Ament: **Frailty in old age; conceptualization and care innovations.** Supervisors: Prof.dr. G.I.J.M. Kempen / Prof.dr. F.R.J. Verhey; Co-Supervisor: Dr. M.E. de Vugt.

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Ingrid M.H. Brands: **The adaptation process after acquired brain injury Pieces of the puzzle.** Supervisors: Prof.dr. C.M. van Heugten / Prof.dr. D.T. Wade, Oxford UK; Co-Supervisors: Dr. S.Z. Stapert / Dr. S. Köhler.

Francesco Risso: **Urinary and salivary S100B monitoring in high risk infants.** Supervisor: Prof.dr. J.S.H. Vles; Co-Supervisors: Dr. D. Gazzolo, Genoa, Italy / Dr. A.W.D. Gavilanes.

Alessandro Borghesi: **Stem and Progenitor Cells in Preterm Infants: Role in the Pathogenesis and Potential for Therapy.** Supervisor: Prof.dr. L. Zimmermann; Prof.dr. B. Kramer; Co-Supervisors: Dr. D. Gazzolo, Genoa, Italy / Dr. A.W.D. Gavilanes.

Claudia Menne-Lothmann: **Affect dynamics; A focus on genes, stress, and an opportunity for change.** Supervisor: Prof.dr. J. van Os; Co-Supervisors: Dr. M. Wichers / Dr. N. Jacobs.

Martine van Nierop: **Surviving childhood new perspectives on the link between childhood trauma and psychosis.** Supervisors: Prof.dr. I. Myin-Germeys / Prof.dr. J. van Os; Co-Supervisor: Dr. R. van Winkel.

Sylvia Klinkenberg: **VNS in children; more than just seizure reduction.** Supervisors: Prof.dr. J. Vles / Prof.dr. A. Aldenkamp; Co-Supervisor: Dr. H. Majoie.

Anouk Linssen: **Considerations in designing an adult hearing screening programme.**

Supervisor: Prof.dr. B. Kremer; Co-Supervisors: Dr. L. Anteunis / Dr. M. Joore.

Janny Hof: **Hearing loss in young children; challenges in assessment and intervention.**

Supervisors: Prof.dr. B. Kremer / Prof.dr. R. Stokroos / Prof.dr. P. van Dijk, RUG; Co-Supervisor: Dr. L. Antheunis.

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Mark Janssen: **Selective stimulation of the subthalamic nucleus in Parkinson's disease; dream or near future.** Supervisors: Prof.dr. Y. Temel / Prof.dr. V. Visser-Vandewalle, Keulen / Prof.dr. A. Benazzouz, Bordeaux, France.

Reina de Kinderen: **Health Technology Assessment in Epilepsy; economic evaluations and preference studies.** Supervisors: Prof.dr. S. Evers / Prof.dr. A. Aldenkamp; Co-Supervisor: Dr. H. Majoie / Dr. D. Postulart, GGZ O-Brabant.

Saskia Ebus: **Interictal epileptiform activity as a marker for clinical outcome.** Supervisors: Prof.dr. A. Aldenkamp / Prof.dr. J. Arends, TUE / Prof.dr. P. Boon, Universiteit Gent, België.

Inge Knuts: **Experimental and clinical studies into determinants of panic severity.** Supervisor: Prof.dr. I. Myin-Germeys; Co-Supervisor: Dr. K. Schruers; Influencing panic.

Nienke Tielemans: **Proactive coping post stroke: The Restored4Stroke Self-Management study.** Supervisors: Prof.dr. C. van Heugten / Prof.dr. J. Visser-Meily, UMC Utrecht; Co-Supervisor: Dr. V. Schepers, UMC Utrecht.

Tom van Zundert: **Improvements Towards Safer Extraglottic Airway Devices.** Supervisors: Prof.dr. A.E.M. Marcus / Prof.dr. W. Buhre / Prof.dr. J.R. Brimacombe, Queensland, Australia / Prof.dr. C.A. Hagberg.

Tijmen van Assen: **Anterior Cutaneous Nerve Entrapment Syndrome Epidemiology and surgical management.** Supervisors: Prof.dr. G.L. Beets / Prof.dr. M. van Kleef / Dr. R.M.H. Roumen / Dr. M.R.M. Scheltinga, MMC Veldhoven.

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Christine van der Leeuw: **Blood, bones and brains; peripheral biological endophenotypes and their structural cerebral correlates in psychotic disorder.** Supervisor: Prof.dr. J. van Os; Co-supervisor: Dr. M. Marcelis.

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Renske Hamel: **The course of mild cognitive impairment and the role of comorbidity.**
Supervisor: Prof.dr. F.R.J. Verhey; Co-supervisors: Dr. I.H.G.B. Ramakers / Dr. P.J. Visser.

The future belongs to those who believe in the
beauty of their dreams.
(Eleanor Roosevelt)